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**SULFONOLIPIDS AS A BIOMARKER FOR THE GENERA *CYTOPHAGA* AND
FLEXIBACTER IN SOIL**

BY

RHAE A. DRIJBER



A THESIS SUBMITTED TO

**THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY**

IN

SOIL SCIENCE

DEPARTMENT OF SOIL SCIENCE

EDMONTON, ALBERTA

SPRING 1993



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15 April 1993

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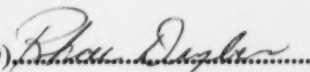
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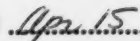
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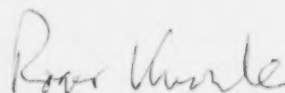
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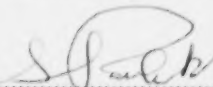
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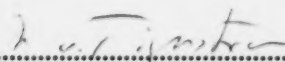
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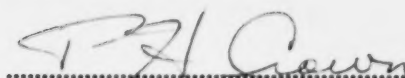
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ABSTRACT

Gliding bacteria of the genera *Cytophaga* and *Flexibacter* contain unusual sulfonolipids, capnoids, that may be potential biomarkers for these bacteria in soil. Three research objectives were developed: (1) to isolate these genera from soil, (2) to evaluate sulfonolipids as a biomarker for them against established criteria, and (3) to apply the biomarker technique to the study of soil worked by the earthworm *Aporrectodea turgida*.

A modification of the cellulose overlay method, the "back-door" method, was developed to isolate and purify cellulolytic cytophagas from enrichment culture using purely a cellulose based medium. Two limitations to the method were noted: (1) the inability of some cellulolytic cytophagas to penetrate the overlay, and (2) penetration of the overlay by noncellulolytic cytophagas to produce cocultures, some of which may be cellulolytic.

Sulfonolipids are major components of *Cytophaga* and *Flexibacter* spp., rivaling phospholipids in abundance. Their suitability as biomarkers was established on the basis that:

(1) Sulfonolipids formed a relatively constant proportion of the biomass of noncellulolytic cytophagas, 15-60 $\mu\text{mol S/g protein}$, which was maintained under differing growth conditions. Cellulolytic cytophagas, however, were differentiated from noncellulolytic cytophagas and *Flexibacter* spp. on the basis of sulfonolipid content, 85-164 $\mu\text{mol S/g protein}$.

(2) Sulfonolipids were extracted completely from whole cells added to soil and did not persist in an extractable form upon cell death.

Three phases of ^{35}S -sulfonolipid dynamics were observed in soil influenced by the activities of *A. turgida*: accumulation, steady state and reduction, and is consistent with the expected pattern for a biomarker sensitive to changes in the biomass of *Cytophaga* and *Flexibacter* spp. in soil. The physical disruption of the soil by the earthworms did not significantly alter either the size or gross composition of the microbial biomass based on phospholipid-P and analysis of fatty acid profiles. Instead, there was a stimulation of subsets of the biomass as illustrated by the altered dynamics of ^{35}S -sulfonolipids.

The specificity of sulfonolipids to the genera *Cytophaga* and *Flexibacter* allowed monitoring of a specific subset of the soil population *in situ*. This is the first study in which an indigenous population has been monitored in soil *in situ*, and it provides a tool to further our understanding of microbial ecology in soil.

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CHAPTER 1

INTRODUCTION

To further knowledge of microbial ecology in soil, a greater understanding of microbial communities as they exist within their natural habitat is essential. This implies not only a better understanding of the microbes themselves and how they interact with one another and with their environment, but also of the physical and chemical makeup of the habitats in which they live and reproduce. Historically, advances in microbial ecology have been made through isolation of microorganisms from soil and characterization of their physiologic potentials under laboratory conditions. The results from these studies are then extrapolated back to the soil environment assuming similarity in function and behavior. To validate these studies and to provide a framework for the functioning of microbial communities in soil, attempts must be made to study soil microorganisms *in situ*. Progress towards this goal has been obtained through the application of transmission electron microscopy to soil micromorphology (Foster 1988). Although, we have a better understanding of the microbial habitat, we are still hampered by a lack of suitable techniques and theories for studying the composition of microbial communities *in situ*. It is towards this goal that my thesis research is directed. To introduce my research objectives and to provide a framework for subsequent chapters, relevant literature was summarized in the following areas: the nature of microorganisms as they exist in soil, methods commonly used to study microorganisms, and an introduction to the genera *Cytophaga* and *Flexibacter*.

The microbial habitat in soil

Soil unites the hydrosphere, atmosphere and geosphere to form a biosphere capable of housing a myriad of life forms from microscopic bacteria to macroscopic plants and animals. The primary particles, sand, silt and clay, form the foundation of a soil structural hierarchy extending from clay microstructures ($< 20 \mu\text{m}$) through microaggregates ($20\text{-}250 \mu\text{m}$) to macroaggregates ($> 250 \mu\text{m}$) (Burns and Davies 1986; Oades and Waters 1991). This structural hierarchy in turn dictates the size and distribution of pores responsible for retention of water (micropores, $0.2\text{-}25 \mu\text{m}$) and exchange of gases (macropores, $> 25 \mu\text{m}$) (Oades 1984; Tisdall and Oades 1982). The

distribution of organic components within this hierarchy, from organo-clay complexes to partially humified plant and animal debris, will determine both the distribution and diversity of microbial communities. The regulation of these communities through the grazing activities of protozoa and nematodes is dependent upon the size and continuity of pores within the structural hierarchy (Bamforth 1985; Vargas and Hattori 1986). Thus, it is this structural hierarchy that ultimately spawns the diversity in microbial habitats, in addition to such recognizable niches as faunal excrement, plant residues, and the rhizosphere and rhizoplane.

Ultrastructural studies have shown the distribution of microorganisms within the soil fabric to be neither random nor uniform: first, microorganisms congregate in pores, and second, microorganisms congregate near metabolizable substrates (Foster 1988; Hattori and Hattori 1976). Microorganisms may also be completely occluded within clay fabrics, often forming the seeds of microaggregates (Foster 1988; Oades and Waters 1991). Using the above hierarchical model of soil structure, two basic types of pores can be distinguished: intraaggregate and interaggregate. Intraaggregate pores are characteristic of microaggregates and can be completely closed, bottle-type or tubes (Hattori and Hattori 1976; Kilburtus 1980). Intraaggregate pores 2 to 6 μm in diameter have the ability to both retain water and comfortably house bacterial cells, and therefore may provide the most favorable habitats for bacteria (Hattori and Hattori 1976). The smaller of these pores (2 μm), especially bottle-type pores with narrow necks, may protect bacteria from predation by protozoa (Bamforth 1985). Similar pores may be found within decaying plant residues as microorganisms congregate within cavities formed in plant tissues through decomposition (Foster 1985). The physical entanglement of microaggregates into macroaggregates by fine roots and fungal hyphae creates interaggregate pores (Miller and Jastrow 1990; Oades and Waters 1991; Tisdall 1991), which although usually air-filled, can provide niches for microorganisms on and within decaying plant and animal debris.

The application of histochemical stains to ultrastructural studies of microorganisms within diverse natural environments established the almost universal presence of the bacterial glycocalyx (Costerton et al. 1981). Newly developed polyanionic stains (specifically ruthenium red), in conjunction with chemicals to stabilize the structure of the bacterial glycocalyx during preparation of specimens for transmission electron microscopy, have been used to demonstrate the existence of such bacterial glycocalyxes in environments as diverse as the rumen, diseased plant and animal tissues, submerged surfaces and sediments of aquatic environments, and medical biomaterials (Costerton et al. 1987). The binding of a single cell to a surface through exopolysaccharide glycocalyx

polymers initiates formation of a biofilm. Subsequent cell division to form microcolonies and the recruitment of other bacterial species produces a microbial consortium housed within a "highly hydrated, predominantly anionic matrix of bacterial exopolymers and trapped extraneous macromolecules" (Costerton et al. 1987). This mode of existence benefits the sessile microbe by retaining a hydrated microenvironment, concentrating nutrients at the interface between the biofilm and the surrounding aqueous phase and providing protection from antimicrobial agents as well as from predators (Costerton et al. 1987). The biofilm serves also as a reservoir of cells able to colonize new environments upon release.

The demonstration of bacterial biofilms in soil is more difficult as habitable surfaces line the walls of pores within aggregates and decomposing residues. Furthermore, the preparation of ultrathin sections from soil is expensive due to the destructive effect sand has on the diamond knives used to slice the soil blocks. Indirect evidence for the existence of bacterial glycocalyxes in soil comes from the extraction of soil polysaccharides which upon hydrolysis contain monosaccharides of bacterial origin (Baldock et al. 1987). Direct evidence for the existence of bacterial glycocalyxes in soils comes from ultrastructural studies of the rhizosphere. Foster (1981) was able to differentiate bacterial polysaccharides from those of plant origin using histochemical stains. Bacterial glycocalyxes have been also detected in pores containing microcolonies within the bulk soil and decaying plant residues (Foster 1985; 1988).

Microorganisms occurring individually or in microcolonies of only a few cells within the bulk soil appear to differ morphologically and possibly physiologically from microorganisms found on or near decomposing residues or plant roots (Foster 1988; Bae and Casida 1973). The terms autochthonous (Winogradsky, cited by Waksman 1932), oligotrophic (Poindexter 1981), ultramicrobacteria (Torrella and Morita, cited in Roszak and Colwell 1987) and dwarf cells (Bae and Casida 1973) have been used to describe the smaller, more coccoid bacteria inhabiting the bulk soil and to distinguish them from the larger zymogenous or copiotrophic bacteria associated with decomposing residues or plant roots. These ultramicrobacteria may be the expression of phenotypic plasticity induced by starvation (Costerton 1988; Morita 1988). They may also be true oligotrophic bacteria which have permanently adapted to the "fast and famine" existence (Poindexter 1981) of the bulk soil by reducing cell size with the resultant increase in surface area per unit volume, as well as by incorporating survival traits common to starved cells. Such traits include reduced endogenous metabolism (Roszak and Colwell 1987), high affinity transport systems and enzymes (Harder et al. 1984) and inducible chemotaxis (Morita 1988).

Thus, microorganisms in soil may exist in a variety of niches, be contained within biofilms or exist freely within the bulk soil solution, and be coping with adverse changes in their microenvironment through the expression of diverse physiological states, such as resting forms or spores. This creates difficulties when selecting a method to enumerate them. The methods commonly employed to enumerate microorganisms in soil can be classified as cultural, microscopic, metabolic, biochemical and genetic (Karl 1986; Sayler et al. 1992).

Measurement of microbial biomass in soil

Cultural methods are based on the ability of microorganisms to reproduce on suitable media. It is virtually impossible to obtain a universally nonselective medium. Thus, cultural methods have an intrinsic selectivity which can be heightened by the addition or deletion of nutrients, antibiotics, metals etc. or by the incubation conditions (Pickup 1991). Further selectivity to the level of species or strain can be achieved through immunofluorescent techniques (Bohlool and Schmidt 1980). For estimations of total biomass, cultural methods are unsuitable due to media selectivity. Enumeration of specific subsets of the microbial population through selective culturing is also constrained by the characteristics of soil microorganisms *in situ*: (1) microorganisms congregate in small pores, many of which are located within microaggregates stable to high speed blending (Bakken 1985); (2) microorganisms are enclosed within glycocalyxes (Costerton et al. 1987; Foster 1988) which may form biofilms tenaciously bound to the walls of pores; and (3) numerous microorganisms in the bulk soil exist as ultramicrobacteria adapted to oligotrophic conditions (Poindexter 1981), thus making them unlikely to reproduce on nutrient media. The reduced recovery of cells added to soil over time has been attributed to the formation of nonculturable but viable cells as the added cells become adapted to the oligotrophic conditions in soil (Pickup 1991). This same loss in culturability could also stem from less efficient removal of cells from the soil as the cells become physically adhered to organo-mineral surfaces through the formation of biofilms.

Enumeration of microorganisms by direct microscopic counting of stained cells typically results in total bacterial counts 10-100 fold greater than culturable methods (Olsen and Bakken 1987). This procedure is not only tedious but is subject to the same constraints as culturable methods since microorganisms have to be removed from the soil to be counted. A further limitation is the objectivity required in differentiating between bacterial cells and soil debris, and between live and dead cells. Metabolic stains, which require uptake by the cell and subsequent transformation, such as fluorescein diacetate

(Lundgren 1981; Söderström 1977), have aided in the latter although the permeability of the cell to fluorescein diacetate may decrease with cell age (Babiuk and Paul 1970). The selectivity of the method can be greatly enhanced through immunofluorescent techniques based on the development of strain specific antibodies labelled with fluorescent dyes, but problems still exist with background autofluorescence and nonspecific staining (Bohlool and Schmidt 1980). Automation of cell counting by laser-based flow cytometry has been successfully used to enumerate aquatic bacteria (Yentsch et al. 1983). Advantages include reduced subjectivity due to the large number of cells measured, short analysis time, increased specificity through immunofluorescent labelling, and cell sizing capabilities (Karl 1986). Enumeration by flow cytometry of an immunofluorescent-labelled *Flavobacterium* P25 added to soil revealed the potential for nonspecific binding of the fluorescein isothiocyanate-labelled antibody to soil particles and interference by soil particulates with the laser detection system (Page and Burns 1991).

Metabolic methods, such as the fumigation method pioneered by Jenkinson (1966), have been widely used to measure total microbial biomass in soil. Metabolic methods are not subject to the same constraints as cultural methods as the microbes do not have to be removed from the soil and recovered in a viable state. The method is based on the mineralization of carbon as CO₂ by surviving organisms or an added inoculum from the microbial biomass after fumigation with chloroform vapor. The mineralized CO₂ is presumed to have arisen from the killed biomass which is being catabolized by the recolonizing microflora. The fraction of the killed biomass C mineralized as CO₂ is assumed to be equal among different soils. A limitation to the technique is the need for a nonfumigated control, and the nature of this control has been the subject of much debate (Voroney and Paul 1984; Shan-Min et al. 1987).

The success of the fumigation method for measuring total microbial biomass C spawned a direct extraction procedure (Powlson and Jenkinson 1976; Vance et al. 1987). This method has the advantage of measuring not only biomass C, but also microbial biomass N (Brookes et al. 1985), S (Saggar et al., 1981) and P (Brookes et al. 1982). An alternative procedure to the fumigation method for total biomass is substrate-induced respiration (Anderson and Domsch 1978). In this procedure, glucose is added to soil to elicit a maximal respiratory response which is then correlated to the total biomass at time zero. Measurements are made within 1 hour after glucose addition to reflect the respiratory response of the initial population. Unlike in the fumigation method, some selectivity can be imposed on the method using selective inhibitors, allowing differentiation between fungal and bacterial biomass (Anderson and Domsch 1978), but such approaches have met with limited success.

Because of the limitations inherent in cultural and microscopic methods and the lack of specificity inherent in metabolic methods for determining microbial biomass, an alternative approach was developed, based on extraction of biochemicals common to the total microbial community or to specific fractions thereof. The success of these methods is highly dependent on their ability to satisfy several criteria as outlined by Jenkinson and Ladd (1981) and reiterated by Karl (1986):

- (1) the constituent is present in all parts of the soil biomass (or subset of the biomass) in the same concentration per cell at all times;
- (2) that it is present only in living organisms and not in dead cells or in the other nonliving parts of the soil organic matter;
- (3) that it can be extracted quantitatively from the soil; and,
- (4) that there is an accurate and precise method for determining it in soil extracts.

The first of these biochemicals to be rigorously tested in soils as a measure of total biomass was adenosine 5'-triphosphate (ATP) (Jenkinson and Ladd 1981). ATP occurs in all living cells and its short half-life in living cells contributes to its rapid loss upon cell death. The extreme sensitivity of the luciferin-luciferase assay allows ATP to be accurately quantified when present in low concentrations. Limitations to the use of ATP stem from problems associated with its extraction from soil and converting amount of extracted ATP to microbial biomass (Jenkinson and Ladd 1981). Furthermore, unless the intracellular content of ATP is stabilized by incubation of the soil at 50-60 % of water holding capacity for several days, the amount of ATP extracted may reflect microbial activity rather than microbial biomass (Nannipieri et al. 1990).

It was anticipated that biochemical markers would become more specific by selecting cell wall components common to broad phylogenetic groups, such as muramic acid (Millar and Casida 1970) and diaminopimelic acid (Durska and Kaszubiak 1980) of the peptidoglycan of prokaryotes, and glucosamine (Swift 1973a,b) of fungal chitin. Unfortunately, the taxonomic diversity within such broad phylogenetic groups essentially ensures nonuniformity in biomarker content, thus preventing accurate assessment of the group's biomass (Karl 1986). Furthermore, as these biochemicals are components of cell wall polymers they cannot be extracted from soil readily but must be released by hydrolysis. The efficiency of the hydrolysis procedure and recovery of the product monomer in the absence of contributions from soil organic matter (Grant and West 1986) and "ghost cells", i.e. cells or hyphae devoid of cytoplasmic constituents, will determine the applicability of the biomarker to soil. Direct observations by transmission electron

microscopy of ghost cells in soils (Lee and Foster 1991) and marine sediments (Moriarty and Hayward 1982) coupled with unrealistic estimations of biomass based on glucosamine (Jenkinson and Ladd 1981; Grant and West 1986; West and Grant 1987), diaminopimelic acid (Grant and West 1986; West and Grant 1987) and muramic acid (Jenkinson and Ladd 1981, based on data in Millar and Casida 1970), make future assessments of the above biomarkers in soils unwarranted.

While the above biomarkers were being developed and evaluated for use in soils and sediments, D. C. White and coworkers were investigating the potential of lipid biomarkers to measure microbial biomass and community structure in sediments (White et al. 1979a,b). Over a period of several years an elaborate scheme (Figure 1.1) for quantifying the microbial community in sediments was developed based on an efficient, single phase, chloroform-methanol extraction (Tunlid and White 1992). The essential features of this protocol are the use of phospholipid-P as a measure of total biomass, the determination of community composition through analysis of profiles of phospholipid-linked fatty acid methyl esters (PL-FAMES), and quantification of the endogenous lipid storage polymer, poly- β -hydroxybutyrate, as an indicator of the nutritional status of the community. The scheme also provides quantification of several other biomarkers, such as ATP and the cell wall polymers muramic acid and diaminopimelic acid, which, as previously discussed, may be unsuitable for use in soils.

Phospholipids are an integral component of the membranes of all living organisms and are not found as storage molecules (Kates 1986). In sediments dominated by prokaryotes, phospholipid-P appears to be a satisfactory measure of total biomass: phospholipids are recovered quantitatively from cells of *Escherichia coli* added to lyophilized sediments (White et al. 1979c); extractable phospholipid-P correlates well with other measures of biomass in subsurface sediments: extractable ATP, rate of DNA synthesis, and direct counts (Balkwill et al. 1988; White et al. 1979c); and, phospholipids are actively metabolized in bacterial monocultures (White and Tucker 1969) as well as in sediments (White et al. 1979c). Uncertainty arises in selection of a conversion factor to equate phospholipid-P to biomass. Although, the phospholipid-P content of 13 diverse bacterial monocultures subjected to a variety of environmental stresses fell within the range: 10-120 $\mu\text{mol-P/g}$ dry mass of cells (tabulated by White et al. 1979a), oxygen status appeared to influence the ratio of phospholipid-P to C in mixed cultures enriched from two marine sediments (Brinch-Iversen and King 1990). While microbial C/N remained constant, the ratio of phospholipid-P to C, which was independent of nutrient concentration and of growth stage as measured by optical density, increased from 100 $\mu\text{mol-P/g C}$ in anaerobic culture to 190 $\mu\text{mol-P/g C}$ in aerobic culture. Unfortunately, no

mention was made of species diversity in the enrichment cultures, leading one to ponder whether changing aeration altered community structure.

The use of PL-FAMES to discriminate between microbial species forms the basis of microbial chemotaxonomy (Tornabene 1985). This discrimination can also be extended to and within the eukaryotic world (Lösel 1988; Wood 1988). The principle types of fatty acids are defined on the basis of carbon chain length, unsaturation and unusual substituents such as methyl branches, hydroxyl groups or cyclopropane rings. Bacterial fatty acids generally fall within the range of C-12 to C-20 (Tornabene 1985), and characteristically contain odd-chain, methyl branched (i.e. iso- and anteiso-branched) and cyclopropane fatty acids, while fungi typically contain saturated even-chain and polyenoic fatty acids (e.g. C-18 with double bonds at C-9 and C-12) (Tunlid and White 1992). Eukaryotes also contain fatty acids longer than C-20, as well as polyenoic ones (Kates 1986).

Application of PL-FAMES to the study of microbial communities in natural and man-made systems is potentially twofold: (1) to use profiles of PL-FAMES to detect changes in microbial community composition or physiological status in response to perturbations of the system (Tunlid et al. 1985; Smith et al. 1986); and, (2) to use "signature" PL-FAMES as biomarkers for specific subsets of the community (Tunlid and White 1992; Federle et al. 1990; Guckert et al. 1985). The latter approach has been successful for validation of PL-FAMES as indicators of microbial community structure where perturbations of the environment have resulted in the desired appearance or disappearance of specific signature PL-FAMES (Dowling et al. 1988; Guckert et al. 1985; Ringelberg et al. 1989; Taylor and Parkes 1985). Signature PL-FAMES have been identified and validated for sulfate-reducing bacteria (Dowling et al. 1988; Taylor and Parkes 1985), methanogens (Hedrick et al. 1991; Mancuso et al. 1990) and methylotrophic bacteria (Nichols et al. 1985; Ringelberg et al. 1989). Fatty acids unique to *Thiobacillus* spp. (Kerger et al. 1986), actinomycetes and fungi may also be suitable signature biomarkers (Tunlid and White 1992).

The accurate measurement of fungal biomass in soil is beset by difficulties due to the diversity of morphological states in which fungi exist, extending from hyphae through fruiting bodies and resting spores to symbiotic associations, and the close association formed between fungal hyphae and their habitat. Microscopic enumeration is generally restricted to hyphal forms, and metabolic stains are required to differentiate live hyphae from dead. Cultural methods tend to favor prolific spore producers, such as *Aspergillus* and *Penicillium* spp., and colonies on agar media often arise from hyphal fragments produced during processing of the soil for plating. Even more significant is the inability

to relate hyphal lengths and colony counts to fungal biomass. Early attempts to use glucosamine, a component of fungal cell walls, as a measure of fungal biomass in soil were unsuccessful as noted above. Ergosterol, the predominant sterol in fungi, was suggested by Seitz et al. (1977) to accurately measure fungal invasion of grain. Recently, the technique has been adapted for use in soils by Grant and West (1986), Johnson and McGill (1990a) and further evaluated by Davis and Lamar (1992) with favorable results. Limitations to the technique stem from inadequate knowledge of the range in ergosterol content among the various fungal taxa and within different morphological states arising through ontogeny and environmental pressures (Davis and Lamar 1992; Johnson and McGill 1990b).

Molecular based genetic systems have the potential for *in situ* monitoring of specific organisms in the environment. The specificity provided equals or surpasses that of immunofluorescent techniques without the laborious production of antibodies or prerequisite removal of the organisms from the soil for microscopic enumeration. Genetic based detection systems can be divided into two classes: (1) nucleic acid hybridization (Sayler and Layton 1990); and (2) bioluminescence (Shaw et al. 1992). Nucleic acid hybridization requires either the release of cells from the soil and subsequent extraction of their DNA, or extraction of the DNA from the cells *in situ*. The specificity of nucleic acid hybridization is dependent on the exclusivity of the DNA sequence chosen: strain, species or genus (Pillai et al. 1992). Increased sensitivity and quantification can be obtained through amplification of the DNA target sequence by polymerase chain reaction, thus enhancing the detection level to a single cell per gram of soil (Steffan and Atlas 1988). Bioluminescence is based on the measurement of light emitted from the target organism containing the bacterial luciferase gene system (Shaw et al. 1992). Applications of this technique to soil include *in situ* localization of *Pseudomonas* spp. in the rhizosphere by autoradiography (De Weger et al. 1991), monitoring the fate of bioluminescent organisms added to soil (Shaw et al. 1992), using organisms containing an inducible luciferase gene system to monitor the movement of pollutants in soil where the pollutant serves as the inducer (De Weger et al. 1991) and to modify the existing system for measuring light emission from cells to allow *in situ* monitoring of cells in the presence of soil (Rattray et al. 1990).

***Cytophagales*: an essential but often neglected component of the soil microbiota**

The following discussion focuses on several genera classified under the order *Cytophagales* as most recently defined in Bergey's Manual of Systematic Bacteriology (1989): *Cytophaga*, *Sporocytophaga*, *Capnocytophaga*, *Chitinophaga*, *Flexibacter* and *Flavobacteria* with low mol % G+C. The order *Cytophagales* can be broadly defined as gram negative rods, often pleomorphic, with microcyst formation confined to two genera: *Sporocytophaga* and *Chitinophaga*. Colonies on nutrient agar are pigmented yellow, orange or red due to nondiffusible carotenoid and flexirubin pigments. Cells are motile by gliding or nonmotile, although demonstration of gliding is often difficult. Many display spreading habit on agar of low nutrient concentration. Carbon and reducing power is gained from organic compounds, i.e. they are chemoorganotrophs, through respiratory (exclusively menaquinones) or fermentative metabolism. Growth is dominantly aerobic, although some species are facultatively anaerobic.

With the exception of *Capnocytophaga* spp., the above genera include species essential to the degradation and turnover of organic residues in terrestrial and marine environments. Not only do these bacteria have the capability to degrade complex biomolecules, such as protein, starch, pectin, cellulose, chitin and alginate, some species interact with the nitrogen cycle through denitrification (Adkins and Knowles 1984). Several species, particularly cellulolytic cytophagas, produce abundant extracellular polysaccharides in liquid culture as well as when grown on solid media. The routine isolation of selected members of *Cytophaga* and *Flexibacter* by plating onto nutrient agar samples taken from soil, fresh-water and marine habitats attests to their ubiquity in the environment (Christensen 1977). Nevertheless, the genera *Cytophaga* and *Flexibacter* also contain nutritionally specialized and fastidious members, such as the cellulose decomposers, which are virtually impossible to isolate on standard nutrient agar (Reichenbach 1989).

The ultrastructure of *Cytophaga* and *Flexibacter* spp. is fairly typical of gram negative bacteria (Reichenbach 1989), although preliminary studies on *Sporocytophaga* Sp13 isolated from earthworm fecal pellets (Chapter 2) and grown on filter paper have uncovered some unusual features (Figure 1.2). The most obvious structural feature is the abundance of internal membranes, which are not mesosomes (Dr. S. Koval, personal communication). Similar internal cytoplasmic membranes have been observed in *Cytophaga columnaris* (Pate and Ordal 1967), *Flexibacter* FS-1 (Poos et al. 1972) as well as in prokaryotes with specialized energy transduction features, such as phototrophs, methylotrophs and nitrifiers (Dr. S. Koval, personal communication). Second, is the

amorphous layer adjacent to the thin peptidoglycan layer in place of the conventional outer membrane bilayer. This may be a result of the fixation procedure used (acrolein/glutaraldehyde) which may not have preserved the outer membrane well (Dr. S. Koval, personal communication). I am presently reviewing other fixation procedures, such as prefixation with osmium or tannic acid, in hopes of verifying whether the amorphous layer is an artifact of fixation.

The lipid profiles of *Cytophaga* and *Flexibacter* spp. have several unusual features which may permit the detection of these bacteria in environmental samples. First, is the presence of a unique sulfonate-containing lipid in the outer membrane of the cell envelope. Collectively, these sulfonolipids are termed capnoids, the most common member being the N-acylated version, N-acylcapnine (Godchaux and Leadbetter 1983). Second, is the abundance of iso-branched 2- and 3-hydroxylated fatty acids, accounting for 30-50 % of the total fatty acids in acid methanolysates of whole cells (Fautz et al. 1979; Fautz et al. 1981). Primary sources of these hydroxylated fatty acids would include the lipid A component of lipopolysaccharides (Wilkinson 1988) and the N-acyl group of sulfonolipids (Godchaux and Leadbetter 1984). Third, are reports of two unusual lipids in *Cytophaga johnsonae*, alpha-N-(3-fatty acyloxy fatty acyl) ornithines (Pitta et al. 1989) and cytolipin, a glycine-containing lipoamino acid analogous to the ornithine amino lipids (Kawazoe et al. 1991). The ornithine-containing lipids of *C. johnsonae* are components of the outer membrane, as are sulfonolipids. It has yet to be established whether the same is true for cytolipin, although from the structural similarity of cytolipin to the ornithine-containing lipids of *C. johnsonae* one might conclude a similar location in the cell envelope.

The presence of hydroxylated fatty acids in the lipopolysaccharides of gram negative bacteria precludes their use as a biomarker for the genera *Cytophaga* and *Flexibacter*, although the iso-branched 2-hydroxy configuration is somewhat novel and could provide a measure of specificity (Fautz 1981). The ornithine- and glycine-containing lipids have only been examined in *C. johnsonae*; thus, their potential as lipid biomarkers is dependent on both their distribution among and exclusivity to the genera *Cytophaga* and *Flexibacter*. Although sulfated lipids are widely distributed in low amounts in both prokaryotes and eukaryotes, sulfonate-containing lipids, with the exception of sulfonoglycosyl diglyceride found in the chloroplasts of plants, have been isolated from only a few organisms (White 1984).

The most abundant and widespread of the sulfonolipids is sulfoquinovosyl diglyceride, found in the chloroplasts of plants and photosynthetic algae (Benson 1963) and in some photosynthetic bacteria (Wood et al. 1965). The sulfonate group of

sulfoquinovosyl diglyceride is located on carbon six of D-quinovose (6-deoxy-D-glucose). Structural variants of sulfoquinovosyl diglyceride found in photosynthetic organisms, the thermoacidophile *Bacillus acidocaldarius* (Langworthy et al. 1976) and the non-photosynthetic marine diatom *Nitzschia alba* (Anderson et al. 1978), a member of the yellow-green algae, are the result of fatty acyl groups esterified to carbons two and three of glycerol. *Nitzschia alba* contains, in addition to sulfoquinovosyl diglyceride, a novel sulfonolipid, deoxyceramide sulfonate (Anderson et al. 1978), which bears a striking similarity to N-acylcapsine. Deoxyceramide sulfonate, however, can be differentiated from N-acylcapsine based on carbon chain length, degree of unsaturation and methyl branching. Furthermore, deoxyceramide sulfonate is a minor component of the lipids of *N. alba*: 0.13 % of the cell dry mass or 0.6% of the cellular lipids (Anderson et al. 1978). This is in contrast to capnoids, which may comprise from 10 to 25 % of the cellular lipids of *Capnocytophaga* spp. (Godchaux and Leadbetter 1980).

Sulfonolipids, specifically 2-amino-3-hydroxy-15-methyl-hexadecane-1-sulfonic acids and their N-acyl derivatives, are structural analogues of sphingosine and ceramides, respectively. Capsine, and its more common form, N-acylcapsine, are illustrated in Figure 1.3. The parent sulfonolipid, capsine, is structurally identical among N-acylcapsines isolated from four different species: *Capnocytophaga* sp. strain P7, *Cytophaga johnsonae* ATCC# 17061, a marine *Cytophaga* sp. strain 7B and a *Flexibacter* sp. strain FS-1 (Godchaux and Leadbetter 1984), and is also identical to capsine originally isolated from *Capnocytophaga gingivalis* strain 30N51 (Godchaux and Leadbetter 1980). Diversity among the N-acylcapsines results from variations in the N-acyl group (Godchaux and Leadbetter 1984), and may provide the organism with phenotypic flexibility, as for example in the synthesis of more polar N-acyl groups in response to growth on a solid substrate (Abbanat et al. 1988).

As most naturally occurring sulfonates are related structurally and metabolically to cysteic acid (alanine-3-sulfonic acid) it was anticipated that cysteic acid would be an important precursor in the biosynthesis of sulfonolipids (Godchaux and Leadbetter 1980). Two independent studies implicated cysteic acid as the immediate precursor in the formation of the sulfonate group in sulfonolipids of *C. johnsonae* (Abbanat et al. 1985; White 1984). Results from both studies are consistent with the condensation of cysteic acid with a fatty acyl coenzyme A (13-methylmyristoyl coenzyme A) to form capsine in a reaction analogous to the condensation of palmitoyl-coenzyme A with serine to form sphingolipids (White 1984).

Gliding motility is found among organisms belonging to diverse taxa: the filamentous blue-green bacteria (e.g. *Anabaena*); the filamentous bacteria, *Beggiatoa*,

Simonsiella, *Saprospira*, *Vitreoscilla*, *Herpetosiphon*, etc.; the fruiting myxobacteria, as well as the genera *Lysobacter*, *Cytophaga* and *Flexibacter* (Henrichsen 1972; Reichenbach 1981; Reichenbach 1989). Early definitions of gliding motility in prokaryotes emphasized a translocation along solid bodies without visible wriggling, contraction or peristaltic motion, the movements being continuous or intermittent and hesitant, with frequent changes in direction (Burchard 1981). Gliding motility can be exhibited by single cells, or more frequently by multicellular associations such as swarms and trichomes (Burchard 1981). The phylogenetic diversity among gliding organisms precludes a common mechanism; however, the machinery must consist of "(a) a biochemical system providing the mechanical energy and (b) a mechanical apparatus translating this energy into a propulsive force...and some agent which mediates the contact of the cell with its substrate" (Reichenbach 1989). The importance of sulfonolipids to this machinery in the genera *Cytophaga* and *Flexibacter* is unknown, except that sulfonolipids are required for gliding motility (Abbanat et al. 1986). Evidence to support this claim comes from studies on nongliding mutants of *C. johnsonae* unable to synthesize sulfonolipids due to a deficiency in cysteic acid production. Provision of cysteic acid to these mutants restores sulfonolipid biosynthesis and also gliding motility.

Research Proposal

Within the literature, there is an apparent dichotomy in our understanding of microbial ecology in soil. While suitable techniques are available for quantifying total soil biota (e.g. metabolic methods) and of individual species or strains of microorganisms (e.g. immunofluorescent and genetic methods), few techniques allow quantification of microbial community structure in soil *in situ*. The narrow range of sulfonolipids in nature, coupled with the absence of capnoids in other gliding bacteria representing the genera *Myxococcus*, *Lysobacter*, *Beggiatoa* and *Herpetosiphon*, as well as from representatives of nine genera of nongliding bacteria (Godchaux and Leadbetter 1983) leads one to conclude that capnoids may serve as a biomarker exclusive to the genera *Cytophaga* and *Flexibacter*. Thus, can this exclusivity be harnessed as a tool to monitor both the presence and activity, by radioisotope labelling of sulfonolipids, of these two genera in soil? Furthermore, will lipid biomarkers provide insight into the ecology of microorganisms in soil worked by the earthworm *Aporrectodea turgida*? To address these questions, my thesis comprises three parts:

1. To isolate representative members of the genera *Cytophaga* and *Flexibacter* from soil using conventional techniques. Existing methods had to be modified to allow purification of cellulolytic cytophagas from enrichment cultures of soil.
2. To evaluate sulfonolipids as a biomarker for the genera *Cytophaga* and *Flexibacter* in soil against established criteria (Jenkinson and Ladd 1981):
 - Does the concentration of sulfonolipids, expressed per gram of cellular protein, vary among different isolates of *Cytophaga* and *Flexibacter* obtained from the same soil environment?
 - Does the concentration of sulfonolipids respond to changes in growth conditions?
 - Do sulfonolipids accurately reflect the population dynamics of *Cytophaga hutchinsonii* during growth on cellulose powder in sand culture?
 - Can sulfonolipids, when added within whole cells, be recovered quantitatively from soil?
 - Do sulfonolipids, when added within whole cells, persist in soil in an extractable form upon cell death?

3. Can lipid biomarkers reveal attributes of community composition and dynamics in soil? Specifically, are lipid biomarkers sensitive enough to detect perturbations in the community caused by the activities of the endogeic earthworm *Aporrectodea turgida*?

- Does the addition of the earthworm *A. turgida* to soil microcosms influence the community structure and dynamics of the soil population as determined by lipid phosphate and PL-FAMES?
- Does the addition of the earthworm *A. turgida* to soil microcosms influence the dynamics of active populations of *Cytophaga* and *Flexibacter* spp. as determined by radiolabelled ^{35}S -sulfonolipids?

This thesis is divided into six chapters and four appendices. In Chapter 2, I report on a technique used to isolate and purify cellulolytic cytophagas from enrichment cultures of soil based on a modification of the cellulose overlay method. The constancy of sulfonolipids as a biomass component within several isolates of *Cytophaga* and *Flexibacter* under differing growth conditions is reported in Chapter 3. The dynamics of sulfonolipids and phospholipids during prolonged growth of a cellulolytic isolate on cellulose powder in cultures containing sterile sand medium, a physical environment intermediate between liquid culture and soil, are reported in Chapter 4. In Chapter 5, the suitability of sulfonolipids as a biomarker for the genera *Cytophaga* and *Flexibacter* in nonsterile soil is addressed. Lipid biomarkers are then used to study microbial community dynamics in soil influenced by the activities of the geophagic earthworm *Aporrectodea turgida*. The merits and potential limitations of sulfonolipids as a biomarker for the genera *Cytophaga* and *Flexibacter* in soil are summarized in Chapter 6 and future uses of sulfonolipids as a tool to extend our understanding of microbial ecology in soil *in situ* are discussed.

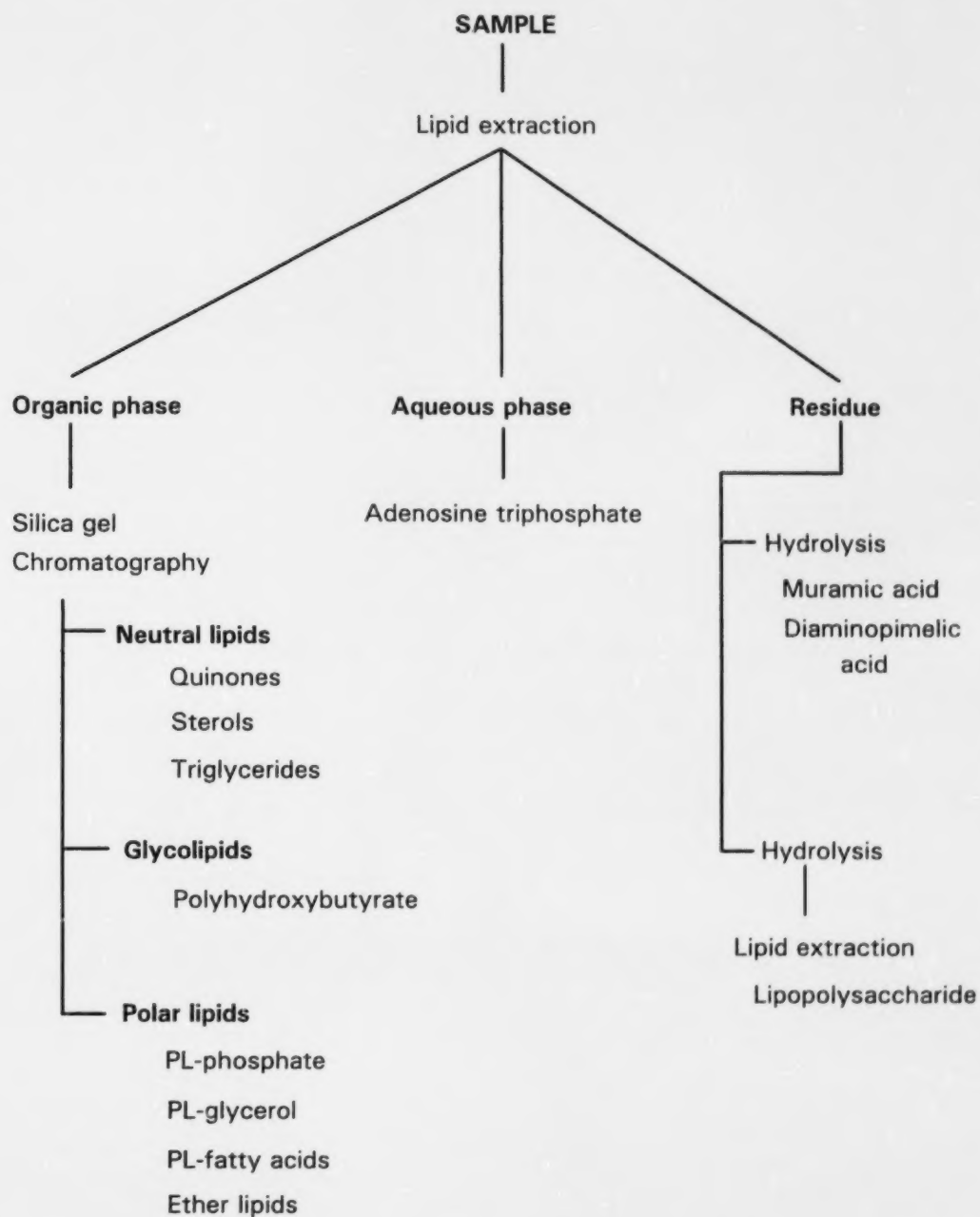


Figure 1.1. Lipid extraction scheme for analysis of biomass and community structure of environmental samples (Tunlid and White 1992). PL = phospholipid-linked.



Figure 1.2. Ultrastructure of *Sporocytophaga* Sp13 as revealed by transmission electron microscopy (TEM prepared by Dr. S. Koval). CM = cytoplasmic membrane, IM = internal membranes, OM = outer membrane.

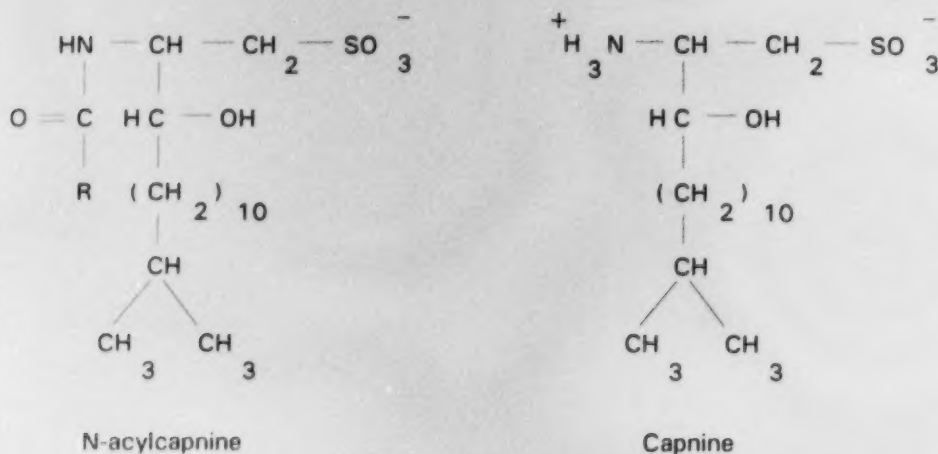


Figure 1.3. Structure of the sulfonolipids, capsine and N-acylcapsine, located in the outer membrane of *Cytophaga* and *Flexibacter* spp. (Godchaux and Leadbetter 1984).

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CHAPTER 2

A MODIFICATION OF THE METHOD USING CELLULOSE OVERLAY AGAR TO ISOLATE AND PURIFY CELLULOLYTIC CYTOPHAGAS FROM ENRICHMENT CULTURE¹

Introduction

This chapter reports on a method, based on the principle of agar penetration by gliding organisms, to purify cellulolytic cytophagas from enrichment cultures. The isolation of cellulolytic cytophagas from soil has been described as a process requiring "skill, considerable patience, and time" (Reichenbach and Dworkin 1981). The proportion of cellulolytic cytophagas to other bacteria in soil is generally small, but can easily be enhanced through enrichment in liquid media or on solid media (Veldkamp 1965; Reichenbach and Dworkin 1981). Antibiotics may be used to restrict the growth of fungi and other noncytophaga contaminants (Mullings and Parish 1980; Warke and Dhala 1968). Further enrichment of the cellulose decomposers is accomplished by several transfers on filter paper placed on agar plates or on cellulose overlay agar (COA). Final purification of the cellulose decomposers has been almost exclusively by streaking onto glucose-mineral salts agar. Although purification of noncellulolytic cytophagas on more complex media containing tryptone and milk has been successful (Christensen and Cook 1972), it is unsuitable for the isolation of cellulose decomposers without the addition of a soluble carbohydrate (Reichenbach 1989).

Attempts to purify cellulolytic cytophagas by streaking are often thwarted if on initial isolation they are unable to utilize glucose (Leadbetter 1974; Sijpesteijn and Fåhræus 1949), or are accompanied by a rapidly growing contaminant with spreading habit.

Due to the requirement of close contact between the *Cytophaga* cell and the cellulose substrate, the COA method results in penetration of the overlay by cellulolytic cytophagas while restricting other noncytophaga contaminants to the surface of the overlay (Reichenbach and Dworkin 1981). Difficulty arises in subsequent removal of the

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cellulose decomposers from within the overlay while avoiding surface contaminants. To overcome this problem I developed a modification of the COA method, for which I coined the term "back-door" method. This modification enabled me to purify the cellulolytic cytophagas within a cellulose based medium. The procedure was then tested on several cellulose enrichment cultures from soil.

Materials and Methods

Enrichment of cellulolytic cytophagas from soil

Soil samples were collected from the Ap horizon of six agricultural soils: two Gray Luvisols, the first a silt loam previously cropped to field pea located at SW18-43-2-W5, the second a loam located at NE25-47-4-W5; a Dark Brown Chernozemic soil of loam texture located at SW18-40-11-W4; a Dark Gray Chernozemic soil, silt loam in texture and previously cropped to barley; and two Black Chernozemic soils, the first a silty clay loam located at NE24-51-25-W4, the second a silt loam located at NW9-49-12-W4. The cellulolytic isolate from the Black Chernozemic soil with silty clay loam texture was obtained from an enrichment culture made from earthworm fecal pellets collected from the Ap horizon. Samples were also taken from a mixed grassland-forest Ah, a garden soil, and a backyard compost pile. Soil and horizon designations follow the Canadian System of Soil Classification (Agriculture Canada Expert Committee on Soil Survey 1987).

Enrichment cultures were set up in 20 mL test tubes containing 10 mL of Dubos mineral medium (Dubos 1928) and a filter paper strip (Whatman No. 1) and covered with Kaputs. The Dubos medium contained 5.9 mM NaNO₃, 5.7 mM K₂HPO₄, 2.0 mM MgSO₄·7H₂O, 6.7 mM KCl and 0.036 mM FeSO₄·7H₂O at pH 7.2. Cycloheximide (25 mg/L) was added to control fungal growth. The inoculum was prepared by mixing in a vortex mixer 1 g soil with 5 mL diluent (0.15 M NaCl, 2.2 mM KH₂PO₄, 4.2 mM Na₂HPO₄). The soil solids were allowed to settle for about 10 minutes before transferring several drops of the soil suspension to each enrichment tube. The enrichment tubes were incubated statically at 22 °C.

After 1-3 weeks of incubation, pale to bright yellow patches appeared on the filter paper immediately above the air-liquid interface. Inoculum from the pigmented zone was then transferred to fresh tubes of medium. Three to four such transfers were usually used

to enrich the cellulolytic cytophagas before streaking the inoculum taken from the pigmented zones onto plates of COA.

The COA was prepared by pouring a thin layer (1-2 mm) of Dubos mineral agar (0.7 % agar, Difco) containing 0.1 % Whatman cellulose powder over a basal layer having the same composition but with 1.5 % agar in standard 100 x 15 mm plastic petri dishes.

After 1-3 weeks of incubation clear zones within the overlay appeared indicating hydrolysis of the cellulose fibers. The "back-door method" was then used to obtain a pure culture of the cellulolytic cytophagas from within the COA.

Isolation and purification of cellulolytic cytophagas

The back-door method

The back-door method extends the principle of agar penetration by gliding organisms. It does so by removing the gliding organisms through the bottom of the plate, thereby preventing recontamination with organisms growing on the agar surface. The method is as follows.

1. Select plates of COA containing zones of cellulose clearing.
2. Stabilize the agar surface over the cleared zone with a sterile 20 x 20 mm cover slip.
3. Invert the petri plate and sterilize the bottom surface with alcohol.
4. Using a flamed, hot scalpel, melt the plastic of the bottom of the plate in the outline of a square (15 x 15 mm) over a selected cleared zone, having some intact fibers visible at the periphery of the cleared zone. Do not sink the hot scalpel into the agar.
5. Pry off the cut square using the scalpel tip. Cover the bottom surface of the plate with a second sterile petri plate lid. Note: several squares can be removed from the plate at one time.
6. Gently remove a cellulose fiber from the edge of the cleared zone with curved, sterile, jeweler's forceps. A dissecting microscope focused on the plane of the cellulose fibers is essential for this step. Note: the tip of the forceps will pass through the basal agar layer first before contacting the cellulose fibers. The key is not to disturb the surface of the overlay during the process otherwise bacteria will be drawn into the overlay by the surface tension of water.

7. Transfer the cellulose fiber to a second cellulose overlay plate by gently touching the tip of the forceps to the gel. Six to eight transfers can be made to one overlay plate.
8. Incubate the plates at 30 °C for two to three weeks or until clear zones appear in the overlay.
9. Repeat the back-door method until there are no surface contaminants present over the cleared zones indicating a pure culture of cellulolytic cytophagas.

The purity of each cellulolytic *Cytophaga* culture in the overlay agar was established by phase contrast microscopy and by streaking onto casitone-yeast extract-glucose agar.

The ability of each cellulolytic *Cytophaga* sp. to utilize glucose and/or cellobiose was tested using Dubos basal mineral agar supplemented with 0.2 % filter-sterilized glucose or cellobiose.

Results and Discussion

Isolation of pure cultures of cellulolytic cytophagas by the back-door method was successful from five of the nine soil samples: two Black Chernozemic samples, the Dark Brown Chernozemic sample, and the garden and compost samples (Table 2.1). In two instances the method allowed more than one cellulolytic isolate to be purified from the enrichment culture. Enrichment cultures from two of the four samples, the Dark Gray Chernozemic and the mixed grassland-forest, were purified of all noncytophaga contaminants leaving a cellulolytic *Cytophaga* sp. in coculture with one or more noncellulolytic cytophagas. All attempts at purifying this coculture by streaking have failed due to extensive swarming of the noncellulolytic *Cytophaga* sp. on all media tested.

The back-door method was unsuccessful for the isolation of cellulolytic cytophagas from enrichment cultures made from both Gray Luvisolic soil samples. Inoculum taken from enrichment cultures of the Gray Luvisolic loam, which demonstrated moderate cellulolysis in the enrichment tubes, failed to produce clearing of cellulose fibers in the overlay plate. Thus, the cellulolysis observed in the enrichment tubes may not have been the result of cellulolytic cytophagas. Alternatively, the overlay environment may not be suitable for the development of all cellulolytic cytophagas, as noted by Reichenbach and Dworkin (1981). Organisms from the Gray Luvisolic silt loam were similar in their

behavior except clearing of the cellulose fibers in the overlay occurred only occasionally upon initial streaking from the enrichment tube. The organisms responsible for clearing of the cellulose fibers, however, could not be transferred to a second overlay plate by the back-door method. Inoculum taken from the clear zones and examined by phase contrast microscopy showed *Cytophaga* cells of aberrant morphologies, i.e. spheroplasts and long rods with clear sacs attached.

Sporocytophagas were isolated from three of the nine soil samples. Microcyst production was not observed in the enrichment tubes, but was readily apparent in the overlay plates for the SiCL Black Chernozemic and garden soils, the cellulose fibers appearing opaque under the dissecting microscope due to microcysts accumulating within the fibers.

The ability of the sporocytophagas to form microcysts was gradually lost on successive culturing in overlay plates. Microcyst production was easily revived by inoculating a basal mineral agar plate containing a wet slurry of cellulose powder on the surface and incubating at 30 °C.

All *Cytophaga* isolates on initial isolation from cellulose cultures were slow to develop on basal mineral agar plates containing glucose or cellobiose; they often required more than 1 week for tiny colonies to develop. Growth on cellobiose was generally more rapid and vigorous than on glucose, especially for the sporocytophagas isolated from the SiCL Black Chernozemic and garden soils. With subsequent transfers on cellobiose or glucose media growth became more rapid and vigorous with colonies appearing in 3-4 days.

Conclusions

In conclusion, the back-door method can be used to both isolate and purify cellulolytic cytophagas from soil samples using a cellulose-based medium as the only source of carbon. The back-door method overcomes the problem of recontamination with organisms from the agar surface when retrieving cellulolytic cytophagas from cellulose overlay agar. Two limitations to the technique were noted. First, the *Cytophaga* cell must be able to penetrate the overlay to remove itself from contaminating bacteria on the surface of the plate. Second, the ability of noncellulolytic cytophagas to penetrate the overlay may produce cocultures of cytophagas, only some of which may be cellulolytic.

Table 2.1. Cellulolytic cytophagas isolated and purified by the back-door method

Soil/Enrichment	No. back-door transfers (days to fiber clearing)	No. of Isolates
Black Chernozemic, SiCL	3 (7-15)	1 ^a
Black Chernozemic, SiL	3 (7-10)	1 ^a
Compost	3 (7-10)	2
Dk. Brown Chernozemic, L	2 (7-10)	2
Dark Gray Chernozemic, SiL	+ (>15)	m
Garden	1 ^b (>7)	1 ^a
Gray Luvisolic, SiL	c	0
Gray Luvisolic, L	d	0
Mixed grassland-forest	+ (>15)	m

Symbols: (+) several transfers made; (m) coculture of two or more *Cytophaga* sp. as observed by phase contrast microscopy, and isolation of the noncellulolytic cytophagas by plating.

Abbreviations: L = loam; SiL = silt loam; SiCL = silty clay loam

^a*Sporocytophaga* sp.

^bCellulose fibers in overlay became opaque due to microcyst formation. Little clearing of cellulose fibers.

^cPoor and erratic clearing of cellulose fibers in the overlay; back-door transfers were not successful.

^dNo clearing or alteration of cellulose fibers in the overlay.

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CHAPTER 3

SULFONOLIPIDS AS A BIOCHEMICAL MARKER FOR THE GENERA *CYTOPHAGA* AND *FLEXIBACTER* IN SOILS: VARIATIONS IN SULFONOLIPID CONTENT AMONG *CYTOPHAGA* AND *FLEXIBACTER* ISOLATES AND WITH CHANGES IN GROWTH CONDITIONS¹

Introduction

The study of microorganisms in natural environments focuses on three aspects: diversity, biomass and activity. Total soil microbial biomass is most frequently estimated using chloroform-fumigation incubation. Diversity of organisms and biomass of selected taxa are traditionally measured by enumeration, such as plating of organisms on nonselective or selective media and direct microscopic counting, and is limited by a lack of specificity and the need for quantitative removal of the organisms from the soil. Although increased specificity can be achieved through immunological methods, enumeration still relies on removal of the target organism from the soil, concentration of the organism by membrane filtration, and finally, direct microscopic counting (Bohlool and Schmidt 1980). An alternative approach, assaying for biochemicals common to the population of interest, has been gaining in popularity in recent years (Tunlid and White 1992; White 1986; Karl 1986; Parkes 1987). The biochemical approach has the advantage of increased specificity, the level of specificity being a function of the biochemical chosen.

The use of lipid biomarkers to gain insight into microbial community structure and dynamics within sedimentary deposits has received considerable attention in the literature (White 1983). The same cannot be said for soil environments, where the application of lipid biomarkers extends only to ergosterol for fungal biomass (Johnson and McGill 1990; Grant and West 1986; West and Grant 1987; Davis and Lamar 1992). Changes in the community structure of the rhizosphere of *Brassica napus* (Tunlid et al. 1985), of soil contaminated with organic surfactants (Federle et al. 1990), and of several long-term agricultural plots (Zelles et al. 1992) have recently been assessed using phospholipid-

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linked fatty acids. This limited application of biomarkers in soil may reflect the unfavorable results obtained for other nonlipid biomarkers such as muramic acid, glucosamine and diaminopimelic acid (Jenkinson and Ladd 1981).

Recently, a unique sulfonate-containing lipid was found in organisms of the genus *Capnocytophaga* (Godchaux and Leadbetter 1980). This compound was shown later to exist in other members of the *Cytophaga-Flexibacter* group, but was absent from gliding bacteria representing the genera *Myxococcus*, *Lysobacter*, *Beggiatoa* and *Herpetosiphon*, as well as from representatives of nine genera of nongliding bacteria (Godchaux and Leadbetter 1983a). Collectively, these sulfonolipids are termed capnoids, the most common member being the N-acylated version, N-acylcapsine. Sulfonolipids are major components of the cell envelope, and constitute up to 25 % of the total cellular lipids (Godchaux and Leadbetter 1983a). This, coupled with the ubiquity of *Cytophaga* and *Flexibacter* spp. in soils and waters (Christensen 1977), led us to consider sulfonolipids as a potential biomarker for these genera in soil.

In their review article on microbial biomass in soil, Jenkinson and Ladd (1981) outlined four criteria essential to the success of biomarker techniques in soils: (1) the constituent is present in all parts of the soil biomass (or subset of the biomass) in the same concentration per cell at all times; (2) it is present only in living organisms and not in dead cells or in the other nonliving parts of the soil organic matter; (3) it can be extracted quantitatively from the soil; and, (4) there is an accurate and precise method for determining it in soil extracts.

In light of these criteria, two questions were posed:

- (1) Does the concentration of sulfonolipids, expressed per gram of cellular protein, vary among species of *Cytophaga* and *Flexibacter* isolated from the same soil environment?
- (2) Does the cellular concentration of sulfonolipids respond to changes in growth conditions?

Materials and methods

Isolation, characterization and cultivation of isolates

Isolation of Cytophaga and Flexibacter from soil

Soil was collected from the Ap horizon of a Black Chernozemic soil located at NE24-51-25-W4. Enrichment cultures for cellulolytic cytophagas were set up in 20 mL test tubes containing 10 mL of Dubos mineral medium (Dubos 1928) and a filter paper strip (Whatman No. 1), and also on Dubos mineral agar overlain by a filter paper (Whatman No. 1). The filter paper on the Dubos mineral agar plates was inoculated with small aggregates of soil, including earthworm fecal pellets and incubated at 30 °C. Inoculum for the enrichment tubes was prepared by mixing with a vortex mixer 1 g soil with 5 mL diluent (0.15 M NaCl, 2.2 mM KH₂PO₄, 4.2 mM Na₂HPO₄). Several drops of soil suspension were then added to each enrichment tube. The enrichment tubes were incubated statically at room temperature (22 °C).

Noncellulolytic bacteria were isolated by dilution plating the soil suspension and the degraded filter paper from the enrichment cultures onto PMYA agar (0.1 % peptonized milk, 0.02 % yeast extract, 0.002 % sodium acetate (Christensen and Cook 1972)), CYG agar (0.3 % casitone, 0.1 % yeast extract, 0.5 % glucose) and Sj agar (Dubos mineral medium, 0.1 % glucose) (Reichenbach and Dworkin 1981). Isolates conforming to the descriptions of *Cytophaga* and *Flexibacter* in Bergey's Manual of Systematic Bacteriology (Reichenbach 1989) were selected. A *Sporocytophaga* (isolate Sp13) was isolated from an enrichment tube containing filter paper using the back-door method (Chapter 2). The other cellulolytic isolates used in this study were obtained similarly from enrichment tubes of filter paper inoculated with soil material taken from a backyard compost pile (isolates CyP2 and CyP4) and a Black Chernozemic soil located at NW9-49-12-W4 (isolate SpV1) (Chapter 2). Soil and horizon designations follow the Canadian System of Soil Classification (Agriculture Canada Expert Committee on Soil Survey 1987). *Cytophaga hutchinsonii* ATCC# 33406 (CH) was donated by R.G. von Tigerstrom, Department of Microbiology, University of Alberta. Diagnostic characteristics of the isolates are listed in Table 3.1.

Cultivation and radiolabeling of bacteria

Isolates were batch cultured in 1 L Erlenmeyer flasks containing 200 mL of medium. The composition of each medium is given in Table 3.2. Radiolabelled ^{35}S -sulfate was added to the medium to give a specific activity of $1.5\text{--}4.4 \times 10^6$ dpm/ μmol ($2.5\text{--}7.3$ kBq/ μmol) sulfate. Flasks were incubated on a rotary shaker (200 rpm) at 22 °C or 29 °C until mid to late log phase (as determined either by absorbance at 660 nm or by protein concentration).

To harvest the cells, the cell culture was poured into a 250 mL centrifuge bottle and centrifuged at 4 °C and $10,000 \times g$ for at least 15 minutes to compact the cells into a pellet. The cell pellet was transferred to a 10 mL Teflon tube and washed once with 7 % trichloroacetic acid and then with water. The washed cells were frozen until extracted for lipids. Extracellular polysaccharides produced by some of the cellulolytic isolates prevented the cells from compacting into a pellet during centrifuging. This was alleviated by adding trichloroacetic acid to the culture medium before centrifuging.

Characterization of isolates

Gliding motility was verified by microscopic examination of wet mounts of cells under phase contrast and by the formation of swarms with characteristic tongue- or flame-like patterns on agar plates with 0.2 % tryptone (Reichenbach 1981). For the cellulolytic isolates, 0.2 % glucose or cellobiose was substituted for the tryptone.

The presence of flexirubin-type pigments was deduced from a color change in the cell mass to red, purple or brown when covered with 20 % KOH, and was confirmed when addition of HCl resulted in the cell mass returning to its yellow color as per Reichenbach et al. (1980).

Isolates were screened for sulfonolipids by extracting cells scraped from the surface of LTY agar containing 0.5 % tryptone, 0.05 % yeast extract, and $100 \mu\text{M}$ $\text{Na}_2^{35}\text{SO}_4$ to give a specific activity of 1×10^6 dpm/ μmol (16.7 kBq/ μmol) sulfate (Abbanat et al. 1988).

Growth on biopolymers was tested by streaking bacterial cells onto plates of mineral salts agar (Dubos 1928) containing the polymer of interest in a thin overlay (0.7 % agar) on top of a 1.5 % agar basal layer.. Starch, casein and gelatin hydrolysis were determined as outlined by Smibert and Krieg (1981). Chitin was prepared by precipitation from concentrated HCl (Reichenbach and Dworkin 1981). Cellulose

powder was obtained from Whatman. Cellulose and chitin hydrolysis was taken to be positive if clearing zones in the overlay were observed.

Changes in cell shape of noncellulolytic isolates during the growth cycle in TGY medium are as described by Hirsch and Reichenbach (1980).

DNA was isolated from cells grown in 1.0 % casitone - 0.2 % yeast extract - 0.1 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Reichenbach and Dworkin 1981) by the method of Marmur as given in the Manual of Methods for General Bacteriology (Johnson 1981). The mol % G+C of DNA from the noncellulolytic isolates was determined from their melting point using DNA from *Escherichia coli* B as a standard. *Cytophaga hutchinsonii* ATCC# 33406 was included as a control, and the mol % G+C of 38.9 agreed with established values (Reichenbach 1989).

Analytical Methods

Protein assays

Protein content of the cell culture was determined on aliquots of the culture medium before harvesting. A 10 mL aliquot of cell culture was centrifuged at 4 °C at 10,000 x g for 10 minutes. Cell-free supernatant was removed with a Pasteur pipette and the resulting cell pellet dissolved in 1 to 5 mL of 1 M NaOH, then heated at 90 °C for 10 minutes. Protein was determined by the method of Lowry et al. (1951) except for cultures containing cellulose powder where the dye-binding method of Bradford (1976) was used. Glucose, released from the cellulose powder during heating in NaOH, increases color development in the Lowry method (Bradford 1976). Bovine albumin (Sigma Chem. Co.) was used as the standard for both assays. Because the molar absorbance of bovine albumin was not consistent between the two assays, protein contents obtained by the dye binding method were converted to a "Lowry protein equivalent" by multiplying by 1.55 for *C. hutchinsonii* grown in glucose (Appendix A).

Lipid extraction procedure

The lipid extraction method of Bligh and Dyer as outlined in Kates (1986) was used with the following modifications: (1) inclusion of 17 M $\text{NH}_3(\text{aq})$ in solvent I (Godchaux and Leadbetter 1980): methanol/chloroform/17 M $\text{NH}_3(\text{aq})$ (10/5/1 Vol./Vol.); (2) addition of 1 M $(\text{NH}_4)_2\text{SO}_4$ during phase separation to prevent emulsion; and, (3) filtration of the

final chloroform extract before evaporating the solvent under nitrogen. Benzene was added during the evaporation step to remove traces of water. The lipid residue was redissolved in 500 μ L chloroform, transferred to a glass vial, capped and stored at -22 °C.

Extraction of sulfonolipids and lipid phosphate from cells by this method was concluded to be quantitative because additional lipids could not be extracted by ultrasonic disruption of the cells. Losses of sulfonolipids during the extraction procedure totaled 1 %.

All solvents were of high purity, glass distilled and filtered (Aldrich Chemical Company, HPLC grade). Chloroform was stabilized with amylenes.

Sulfonolipid assay

The cellular content of sulfonolipids was determined from the radioactivity in sulfonolipids and the specific activity of sulfate in the growth medium. Aliquots (10-25 μ L) of lipid extract were added to a 20 mL scintillation vial, the solvent evaporated, and the residue dissolved in 0.5 mL sodium dodecyl sulfate (0.1 %) and 10 mL Scintiverse II (Fisher Chemical Company). Disintegrations per minute (dpm) were determined by efficiency tracing (Ishikawa et al. 1984) on a Packard 2000A Liquid Scintillation Analyzer.

For isolates grown in the presence of tryptone and inorganic sulfate (TGY medium), the total cellular content of sulfonolipids was determined by reaction with azure-A dye (Kates 1986) using bovine sulfatide as the standard. The proportion of sulfonolipid-S originating from inorganic sulfate in the growth medium was determined from the radioactivity in the sulfonolipids. Contributions to sulfonolipid-S from S originating in tryptone were calculated by difference. Phospholipids, which can react with azure-A, were removed from the lipid extract by mild alkaline hydrolysis. This procedure does not affect the sulfonolipids (Godchaux and Leadbetter, 1980; unpublished results). Because the molar absorbance of bovine sulfatide and sulfonolipids may differ, the azure-A method was calibrated using sulfonolipids extracted from isolates grown in the presence of inorganic sulfate only (Appendix B). Addition of ^{35}S -sulfate to the growth medium allowed the actual sulfonolipid content of the isolates to be determined by isotope dilution.

Sulfonolipid hydrolysis

A 50-100 μL aliquot of lipid extract was hydrolyzed in 1 M HCL-10 M H_2O in methanol (Godchaux and Leadbetter 1983a). The hydrolyzed lipid residue was dissolved in solvent I.

Thin-layer chromatography and autoradiography

Thin-layer plates (Silica gel G; Universal Scientific Inc.) were spotted with ^{35}S -labelled lipid extracts and developed with chloroform-methanol-7 M $\text{NH}_3(\text{aq})$ (70:30:5 Vol./Vol.) (Godchaux and Leadbetter 1980). A lipid extract, containing ^{35}S -labelled sulfonolipids extracted from *C. hutchinsonii*, was used as a chromatography standard. The dried plates were placed in contact with X-ray film (Kodak XRP-5) for 2-4 days. The film was developed as recommended by the manufacturer. As little as 100 dpm could be detected. Inorganic sulfate (which remains at the origin on the plate) accounted for < 1 % of the total radioactivity in the lipid extracts. The remaining radioactivity was present as sulfonolipids.

Spray reagents were applied to the plates to detect phospholipids and lipids containing free amino groups (Kates 1986).

Mass spectrometry

Sulfonolipids isolated from *C. hutchinsonii* were analyzed by mass spectrometry. *C. hutchinsonii* was cultured in NGY medium to late log phase then harvested by centrifugation as outlined previously. The N-acylcaptines were extracted and purified from 11 g of cells by the method of Godchaux and Leadbetter (1983b). N-acylcaptine was hydrolyzed in 1 M HCL-10 M H_2O in methanol (Godchaux and Leadbetter 1983a). The fatty acids released from amide linkage with N-acylcaptine were removed in hexane. Captine, retained in the methanol phase, was deprotonated by the addition of aqueous ammonia, then extracted into chloroform upon the addition of chloroform and water. The mass spectrum of N-acylcaptine (isolated as the sodium salt) and captine (isolated as the ammonium salt) was obtained by negative ion fast atom bombardment (FAB(-)) in glycerol and Cleland matrices, respectively, at the University of Alberta, Mass Spectrometry Laboratory.

Lipid phosphate assay

A 10-50 μ L aliquot of lipid extract was pipetted into a acid-washed Pyrex test tube and the chloroform was evaporated. Perchloric acid was added to the lipid residue and the tube heated overnight in a sand bath set at 135 °C. Released phosphate was determined by the method of Bartlett as described by Kates (1986).

Inclusion of aqueous ammonia in solvent I of the extraction procedure for lipids did not affect the recovery of lipid phosphate and sulfonolipids in the final extract from which it was inferred that the basicity of the solvent was not strong enough to cause hydrolysis of the phospholipids, in agreement with Cotton and Wilkinson (1980).

Results

Characterization of isolates

Enrichment culture and dilution plating led to the isolation of seven noncellulolytic and one cellulolytic species (Sp13) from the soil sample. The diagnostic features and genus affiliations of these isolates plus three additional cellulolytic isolates are given in Table 3.1.

Gliding motility, both in wet mounts viewed under phase contrast microscopy and on low nutrient agar plates, was observed for five of the seven noncellulolytic isolates and all cellulolytic isolates. Gliding motility could not be demonstrated for two of the isolates, G1 and CyG3, which also did not exhibit a cellular shape change in tryptone medium. The taxonomic position of isolate G1 is uncertain as the mol % G+C is consistent with *Flexibacter* spp.; however, isolate G1 consistently formed short to medium length rods ($<1 \times 3-4 \mu\text{m}$) in both AGY and TGY medium, media known to promote the formation of long thread-like cells typical of *Flexibacter* spp. Isolate G1 bears a resemblance to a succinoglycan-decomposing bacterium, *Cytophaga arvensicola*, which also exists as short rods, fails to exhibit gliding motility, shows a positive reaction for flexirubins, cannot hydrolyze starch or gelatin and has a mol % G+C of 46 (Oyaizu et al. 1982; Reichenbach 1989). Further study of isolate G1 is necessary to determine its taxonomic position.

Prescreening of isolates grown on agar supplemented with ^{35}S -sulfate for sulfonolipids showed on thin-layer chromatography and autoradiography one or two ^{35}S -lipid zones in the region of N-acylcapsine (Godchaux and Leadbetter 1980). The

deacylated form capnine, which is characterized by an R_f of 0.3-0.35, was not detected from any of the isolates. Two of the isolates, CyS1 and CyS2, contained a third, less intense ^{35}S -lipid zone which was more polar than the other zones, but was not ninhydrin reactive.

Sulfonolipid identity

The identity of the sulfonolipids was verified by thin-layer chromatography and autoradiography of nonhydrolyzed and acid hydrolyzed lipid extracts. The nonhydrolyzed ^{35}S -labelled lipids were consistent in R_f (0.6-0.67) and staining behavior with N-acylcapnine (Godchaux and Leadbetter 1980). Upon acid hydrolysis the ^{35}S -labelled lipids chromatographed as a single zone which was ninhydrin reactive and had an R_f (0.3-0.35) characteristic of the deacylated sulfonolipid, capnine (Godchaux and Leadbetter 1980). The N-acylcapnines were the sole ^{35}S -labelled lipids present in the isolates under all growth conditions.

The mass spectrum of the N-aminosulfonates isolated from *C. hutchinsonii* is given in Figure 3.1a. The molecular ion was readily identified at $m/z = 642$ which was slightly higher than the reported range in mass for N-acylcapnines (Godchaux and Leadbetter 1984). Two other abundant ions were observed at $m/z = 390$ and 374 , the latter in agreement with the molecular ion of capnine, $m/z = 374$ (Figure 3.1b). The mass difference of 269 between $m/z = 642$ and capnine is consistent with a 17C fatty acyl moiety containing a hydroxyl group. This fatty acyl group was subsequently identified as *i*C17:0 (2-OH) by gas chromatography-mass spectrometry (Appendix C). The molecular ion observed for capnine isolated from *C. hutchinsonii* is 24 mass units greater than reported by Godchaux and Leadbetter for capnine isolated from *Capnocytophaga* (Godchaux and Leadbetter 1980) and four other species (Godchaux and Leadbetter 1984) and contains a *trans*-double bond (Appendix C). Additional studies on structure of the N-aminosulfonates of *C. hutchinsonii* are in progress to locate the source of the additional mass units contained within the capnine molecule.

Sulfonolipid and phospholipid content: noncellulolytic isolates

The ratio of sulfonolipid-S to protein in the noncellulolytic isolates of *Cytophaga* and *Flexibacter* varied more among isolates than within an isolate under conditions of

differing temperature and media composition (Figure 3.2) The ratio of sulfonolipid-S to protein was highly reproducible for isolates CyS1 and FxG4 in AGY medium at 22 °C when batch cultured several months later (means not significantly different, $p=0.05$). AGY medium at 22 °C was chosen as the standard medium to compare the sulfonolipid content among the isolates because it supported the growth of all but one of the isolates, CyS2. Only two of the isolates grew in basal mineral medium (NG or NGY) with glucose and nitrate as the carbon and nitrogen sources, respectively. The ratio of sulfonolipid-S to protein of *Flexibacter* isolates grown in AGY medium spanned a narrow range: 48-60 $\mu\text{mol-S/g}$ cell protein. In contrast, *Cytophaga* isolates grown in AGY medium had ratios of sulfonolipid-S to protein which varied from 14-61 $\mu\text{mol-S/g}$ cell protein. An increase in the growth temperature of AGY medium from 22 °C to 29 °C increased the ratio of sulfonolipid-S to protein by 15 to 35 % in three of the four isolates tested. The sulfonolipid-S to protein ratio of the fourth isolate, FxE1, was reduced by 11 %.

Isolate CyS2, which required a more complex medium for growth, was similar in sulfonolipid concentration to isolate CyS1. The ratio of sulfonolipid-S to protein of isolate FxG4, however, was considerably reduced in tryptone medium. Inorganic sulfate was the primary source of sulfonolipid-S in isolates CyS2 and FxG4, at 89 % and 92 % respectively. Contributions from inorganic sulfate reached 55 % for isolate CyS1, the remaining 45 % of sulfonolipid-S originated from tryptone.

The phospholipid-P content of the noncellulolytic isolates mirrored the variation in sulfonolipid-S content with two exceptions, isolates CyG1 and FxG4 (Figure 3.3). The proportionately lower ratio of phospholipid-P to protein in isolates CyG1 and FxG4 is reflected in ratios of sulfonolipid-S to phospholipid-P (0.45-0.56) slightly exceeding the values for the other isolates (0.22-0.42) (Figure 3.4).

Sulfonolipid and phospholipid content: cellulolytic isolates

The sulfonolipid profiles of the cellulolytic isolates grown medium containing cellulose powder were distinct from the noncellulolytic isolates (Figure 3.5). Ratios of phospholipid-P to protein were within the boundaries observed for noncellulolytic isolates (111-176 $\mu\text{mol-P/g}$ protein); however, ratios of sulfonolipid-S to protein were consistently elevated, causing a doubling in the ratio of sulfonolipid-S to phospholipid-P (0.71-0.98).

Growth of *C. hutchinsonii* on glucose in NGY medium did not change the content of sulfonolipids (172.14 $\mu\text{mol-S/g}$ protein; $\text{sd}=14.14$; $n=12$); however, the content of phospholipid-P increased (198.77 $\mu\text{mol-P/g}$ protein; $\text{sd}=6.82$; $n=12$) giving rise to a decreased ratio of sulfonolipid-S to phospholipid-P (0.87; $\text{sd}=0.05$; $n=12$).

Discussion

The limits established by Godchaux and Leadbetter (1983a) for the cellular content of sulfonolipids in organisms belonging to the genera *Cytophaga* and *Flexibacter*, required further refinement before sulfonolipids could be used as a biomarker for these organisms in soil. First, expression of the cellular content of sulfonolipids on the basis of wet mass of cells was replaced by protein, a more reliable indicator of biomass. Second, the cellular content of sulfonolipids was compared to the content of phospholipids, a major membrane component (Kates 1986). And third, the constancy of sulfonolipids as a biomass component was addressed under conditions of differing growth temperature and media composition.

The concentration of sulfonolipids, expressed per gram of cellular protein, separated the isolates into two groups, which coincided with their ability to hydrolyze cellulose. The sulfonolipid concentration of the noncellulolytic isolates grown under standard conditions (AGY medium, 22 °C), with the exception of isolate CyG3, fell within a narrow range (40-60 $\mu\text{mol-S/g}$ cell protein). The low concentration of sulfonolipids in isolate CyG3 was reflected also in low concentrations of phospholipids; however, the ratio of sulfonolipids to phospholipids was comparable to the other *Cytophaga* isolates. The range in sulfonolipid content per gram wet mass of cells (data not shown) for the noncellulolytic isolates was within the 2-16 $\mu\text{mol-S/g}$ cells (wet mass) reported by Godchaux and Leadbetter (1983a). Expression of the sulfonolipid content on a protein basis has been reported only for *C. johnsonae* (ATCC 43786), 27-28 $\mu\text{mol-S/g}$ protein in either defined or complex media (Gilmore et al. 1989), which compared well with the noncellulolytic isolates in this study.

The phospholipid-P concentration of isolates of *Cytophaga* and *Flexibacter* subjected to differing growth conditions fell between 59 and 198 $\mu\text{mol-P/g}$ cell protein. Assuming a typical bacterial cell is 55 % protein by dry mass, cells of *Cytophaga* and *Flexibacter* contain 32 to 109 $\mu\text{mol-P/g}$ dry mass of cells. This agrees with the range of 10-120 $\mu\text{mol-P/g}$ dry mass of cells tabulated by White et al. (1979) for 13 diverse bacterial monocultures subjected to a variety of environmental stresses.

The concentration of sulfonolipids within an isolate changed in response to growth temperature and media composition; however, the magnitude of the change was generally small and inconsistent in direction. Thus, with the exception of isolates CyG3 and FxG4 in TGY medium, the sulfonolipid concentration was maintained between 40-60 $\mu\text{mol-S/g}$ cell protein under differing growth conditions. The decrease in sulfonolipid concentration of isolate FxG4 in TGY medium was not mirrored by changes in phospholipid concentration, however, variation in sulfonolipid concentration was reflected in varying ratios of sulfonolipid-S to phospholipid-P. *C. johnsonae* ATCC 43786 maintained a constant concentration of sulfonolipids in response to changes in medium composition (Gilmore et al. 1989) and to growth on a solid substrate (Abbanat et al. 1988), although in the latter there was a change in the distribution of sulfonolipid species. Growth of isolates CyS1 and CyS2 on tryptone-yeast extract agar induced the synthesis of an additional sulfur-containing lipid which may correspond to the more polar variant of N-acylcapsine observed in surface-grown cells of *C. johnsonae* (Abbanat et al. 1988).

When given two sources of sulfur in the growth medium, isolates CyS2 and FxG4 preferentially used inorganic sulfate over tryptone for sulfonolipid biosynthesis. Under identical growth conditions, the third isolate, CyS1, incorporated approximately equal amounts of sulfur from both sources into sulfonolipids. This was contrary to the findings of Gilmore et al. (1989) in which *Cytophaga johnsonae* ATCC 43786 used inorganic sulfate primarily for sulfonolipid synthesis and reduced organic-S for protein synthesis. Thus, the mechanisms of sulfate assimilation and incorporation into sulfonolipids may not be universal among *Cytophaga* and *Flexibacter* spp.

The sulfonolipid concentration of the cellulolytic isolates (85-164 $\mu\text{mol-S/g}$ cell protein) was at least double that found for the noncellulolytic isolates. This difference could not be due to abnormally low contents of cell protein as the ratios of phospholipid-P to cell protein were similar to the noncellulolytic isolates. A change in carbon source from glucose to cellulose powder did not affect the sulfonolipid concentration of *C. hutchinsonii*; therefore, it is unlikely that the other cellulolytic isolates would respond differently. In defined medium with glucose, *C. hutchinsonii* contained 20 $\mu\text{mol-S/g}$ wet mass of cells, which is in excess of the range 2-16 $\mu\text{mol-S/g}$ wet mass of cells reported by Godchaux and Leadbetter (1983a). The high concentration of sulfonolipids found in this study for the sporocytophagas Sp13 and SpV1 was contrary to the findings of Godchaux and Leadbetter (1983a) for *Sporocytophaga myxococcoides*. This may reflect the problem of reporting sulfonolipid concentration on the basis of wet mass of cells, as the

authors did allude to the presence of abundant extracellular polysaccharides in the growth medium.

The division of the isolates into two groups based on sulfonolipid concentration was especially apparent from the ratios of sulfonolipid-S to phospholipid-P. The noncellulolytic isolates all had ratios below 0.6, whereas the cellulolytic isolates had ratios above 0.7. This dichotomy in sulfonolipid content between cellulolytic cytophagas and other *Cytophaga* and *Flexibacter* spp., in terms of both cellular protein and phospholipid-P, supports the proposal by Reichenbach (1989) to reserve the genus *Cytophaga* for cellulose decomposers related to *C. hutchinsonii*.

Does this dichotomy in sulfonolipid content pose a limitation on the use of sulfonolipids as a biomarker for the genera *Cytophaga* and *Flexibacter* in soil? To address this question the likelihood of sampling a portion of the soil containing a population consisting mainly of cellulolytic cytophagas must be examined. If the soil sample is assumed to contain equal numbers of each isolate, including the *Sporocytophaga* Sp13, the mean concentrations of sulfonolipids, as given in figures 3.2 and 3.5, can be used to construct a sampling distribution of the means. Assuming a normal distribution, the sampling distribution would have a mean of 52.6 $\mu\text{mol-S/g}$ cell protein and a standard deviation of 22.5. Thus, the probability of sampling a portion of the soil containing dominantly isolate Sp13 is less than 5 %. This probability is reduced even further when the size of soil sample required for sulfonolipid analysis is considered: a minimum of 2-3 g using isotope dilution techniques as described in Chapter 5. All of the above isolates could be isolated from a 1 g sample of soil (see methods section).

In an extreme situation where the contribution of isolate Sp13 is increased to 50 % of the total *Cytophaga* and *Flexibacter* population, the mean of the sampling distribution of the means (71.3 $\mu\text{mol-S/g}$ cell protein) is still within the initial standard deviation of the mean calculated when all isolates were present in equal amounts. To fall outside this range, isolate Sp13 would have to comprise 60 % of the population. In light of the difficulty encountered in purifying enrichment cultures for cellulolytic cytophagas from noncellulolytic cytophagas (Chapter 2), a dominance of isolate Sp13 in soil would be unlikely. To quote Poindexter and Leadbetter (1986), "only in extremely rare, and often then only transient, situations does a single type of bacterium occupy a natural habitat alone". In contrast, in habitats such as cattle manure (Godden and Penninckx 1984) and sewage sludge under aerobic conditions (Güde 1980), cellulolytic cytophagas may however be dominant.

Conclusions

Sulfonolipids, in terms of both cellular protein and phospholipid-P, are major cell components of *Cytophaga* and *Flexibacter* spp. Two criteria essential to the success of sulfonolipids as a biomarker for these organisms in soil were met: (1) sulfonolipid concentration among the noncellulolytic isolates, with the exception of isolate CyG3, spanned a narrow range (40-60 $\mu\text{mol-S/g}$ cell protein), and (2), this range in sulfonolipid concentration was maintained under the range of growth conditions studied here. Cellulolytic cytophagas could be differentiated from other *Cytophaga* and *Flexibacter* spp. on the basis of sulfonolipid content, 85-164 $\mu\text{mol-S/g}$ cell protein. For the mean sulfonolipid content to deviate from the expected range in sulfonolipid concentration for soil isolates, cellulolytic cytophagas would have to comprise at least 60 % of the population of *Cytophaga* and *Flexibacter*.

Table 3.1. Characteristics of isolates of *Cytophaga* and *Flexibacter* from soil and earthworm fecal pellets

Isolate	Gliding	Gram	KOH	Sulfono-	Polymer ^s used ¹	Shape change ²	mol % G+C	Genus
		reaction		lipids				
Noncellulolytic								
CyS1	+	-	+	+	cn,st,cs	1	36.3	<i>Cytophaga</i>
CyS2	+	-	+	+	st,cs	1	36.5	<i>Cytophaga</i>
CyG3	-	-	+	+	st,cs	0	38.3	<i>Cytophaga</i>
FxE1	+	-	+	+	-	2	nd ³	<i>Flexibacter</i>
FxE2	+	-	+	+	cs	2	43.6	<i>Flexibacter</i>
FxG4	+	-	+	+	cs	2	48.0	<i>Flexibacter</i>
G1	-	-	+	+	-	0	48.2	?
Cellulolytic								
CyP2	+	-	+	+	cel	nd	nd	<i>Cytophaga</i>
CyP4	+	-	+	+	cel	nd	nd	<i>Cytophaga</i>
SpV1	+	-	+	+	cel	nd	nd	<i>Sporocytophaga</i>
Sp13	+	-	+	+	cel	nd	nd	<i>Sporocytophaga</i>

Symbols: (+) positive; (-) negative.

1. Biopolymers abbreviated as follows: starch = st, casein = cs, gelatin = gt, cellulose = cel, chitin = cn.

2. Shape change in TGY medium: 0 = no cell shape change, 1 = short rods change to long rigid cells in old cultures, 2 = long thread-like cells in young culture fragment to short rods in old cultures.

3. nd = not determined

Table 3.2. Composition of media used to culture isolates of *Cytophaga* and *Flexibacter*.

Name	Basal salts ¹	Nitrogen source ²	Carbon source ³	Yeast extract µg/mL	MgSO ₄ ·7H ₂ O mM	K ₂ HPO ₄ mM
NG	+	N	G	-	-	-
NGY	+	N	G	50	-	-
AGY	+	A	G	50	-	-
NCY	+	N	C	50	-	-
TGY	-	T	G	50	1.0	2.85

Symbols: (+) present; (-) absent.

1. Basal salts medium: 5.7 mM K₂HPO₄; 6.7 mM KCl; 1.0 mM MgSO₄·7H₂O; and 0.036 mM FeSO₄·7H₂O
2. Nitrogen source: N: 5.9 mM NaNO₃; A: amino acids in similar proportions to casein hydrolysate with the omission of sulfur containing amino acids (Guirard and Snell 1981), 2 g/L; T, 2 g/L tryptone (Difco).
3. Carbon source: G: 11.1 mM glucose; C: 1 g/L cellulose powder (Whatman).

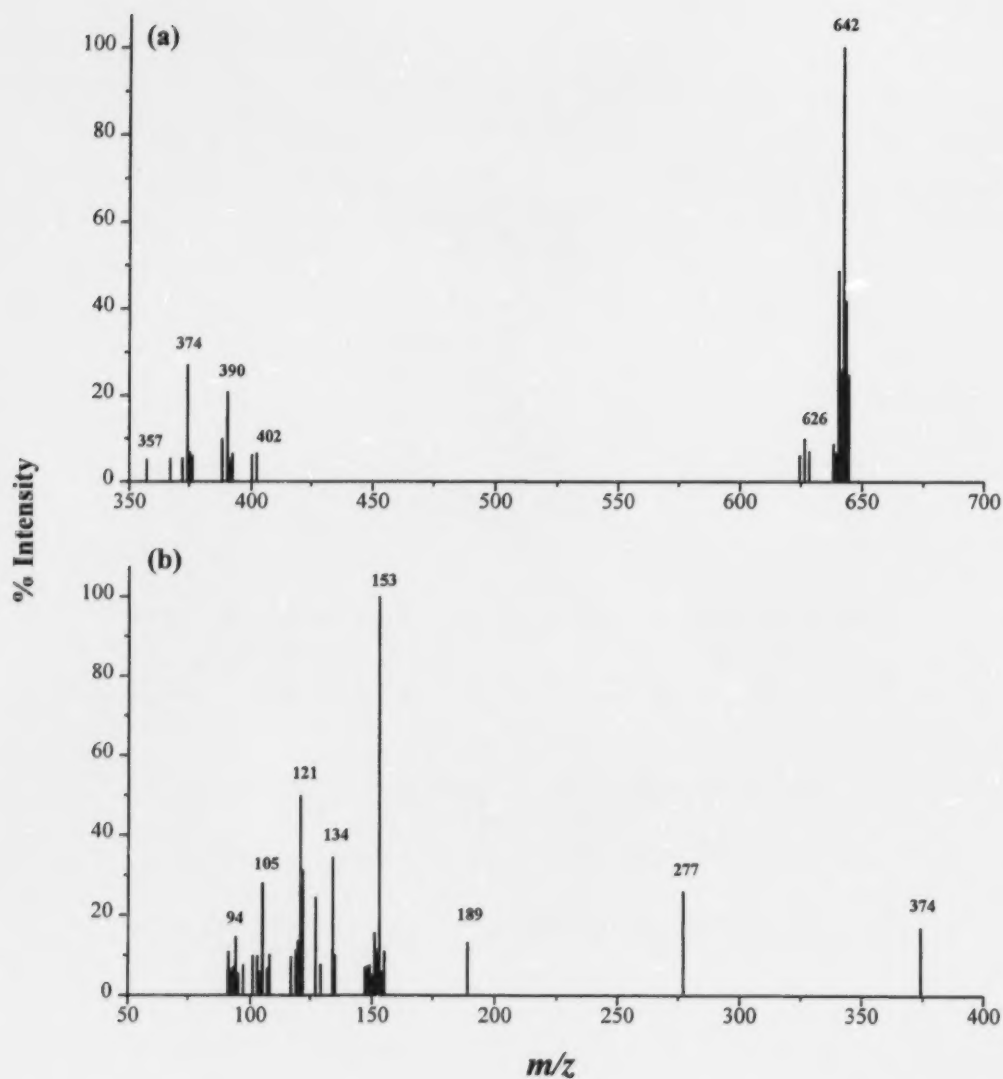


Figure 3.1. Mass spectrum obtained by negative ion fast atom bombardment of sulfonolipids isolated from *Cytophaga hutchinsonii*. (a) N-acylcapsine (sodium salt); (b) capsine (ammonium salt). Mass fragments at < 5 % of maximum intensity not shown.

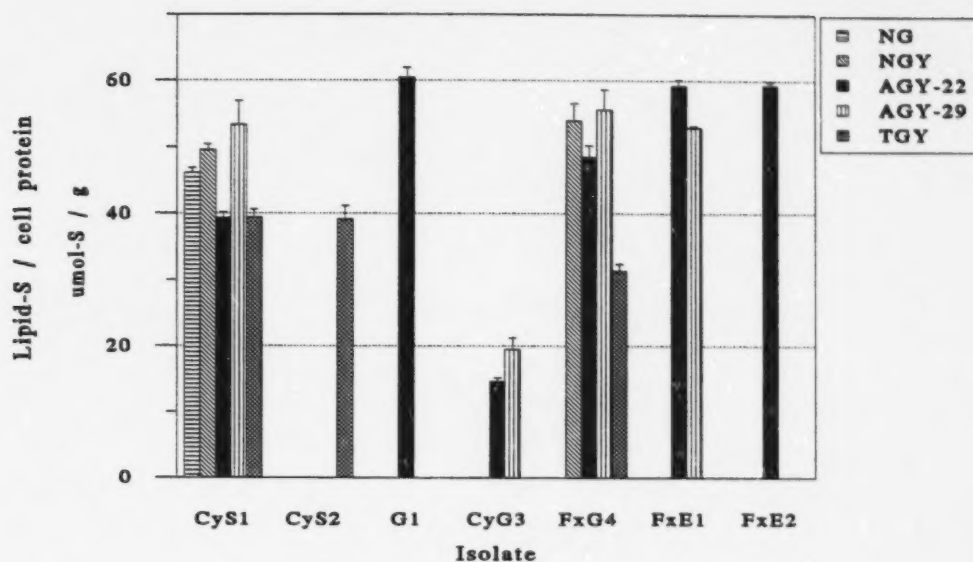


Figure 3.2. Sulfonolipid-S content of noncellulolytic isolates under differing growth conditions. Media composition given in Table 3.2. Bars are sample standard deviations of the mean, $n = 4$; Cy = *Cytophaga*, Fx = *Flexibacter*.

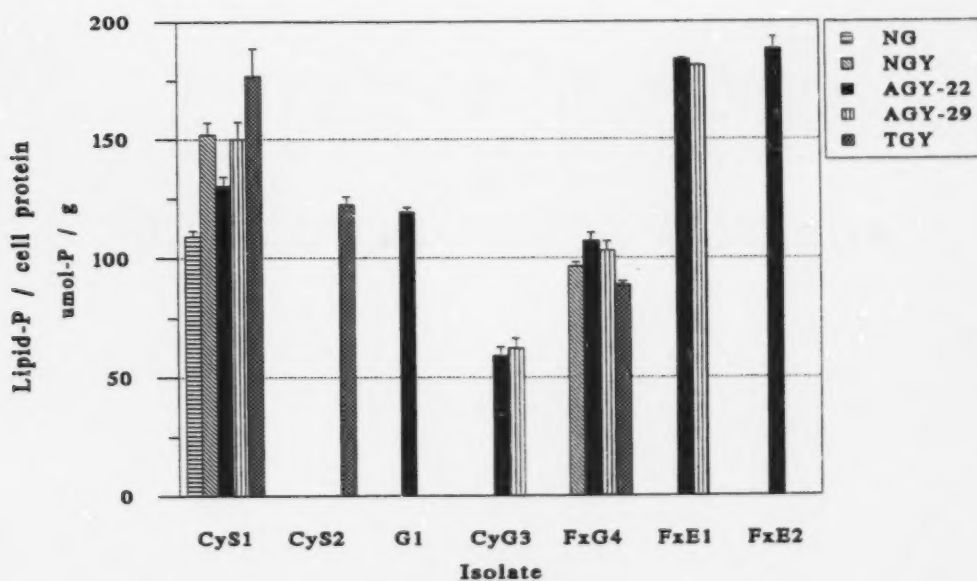


Figure 3.3. Phospholipid-P content of noncellulolytic isolates under differing growth conditions. Media composition given in Table 3.2. Bars are sample standard deviations of the mean, $n = 4$; Cy = *Cytophaga*, Fx = *Flexibacter*.

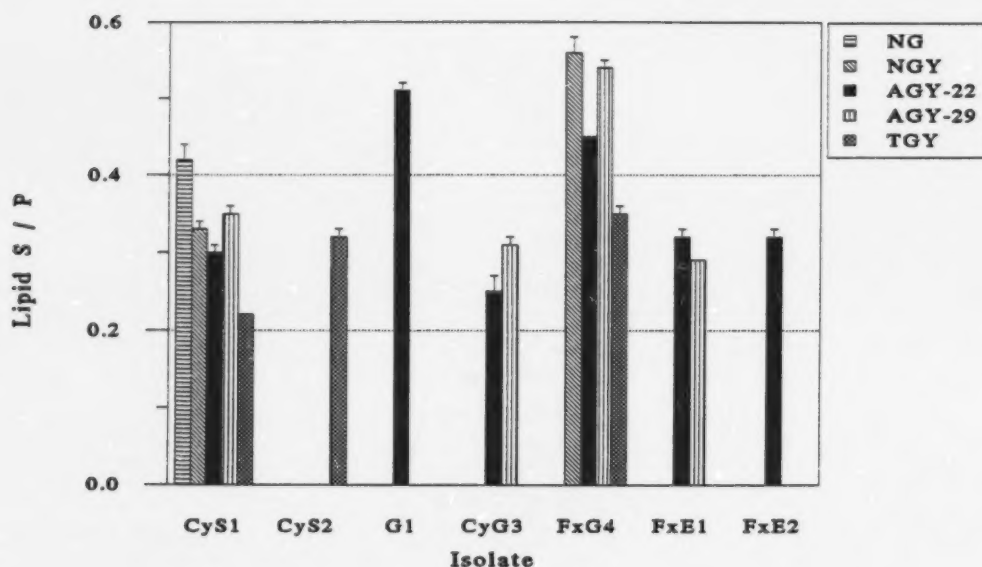


Figure 3.4. Ratio of sulfonolipid-S to phospholipid-P of noncellulolytic isolates under differing growth conditions. Media composition given in Table 3.2. Bars are sample standard deviations of the mean, $n = 4$; Cy = *Cytophaga*, Fx = *Flexibacter*.

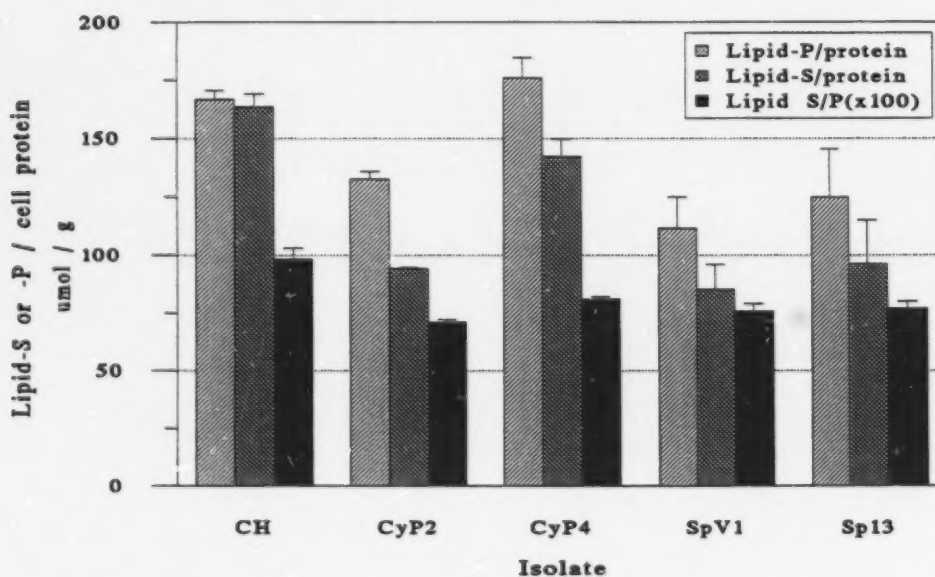


Figure 3.5. Sulfonolipid-S and phospholipid-P content of cellulolytic isolates grown in medium containing cellulose powder. Bars are sample standard deviations of the mean, $n = 4$; CH = *Cytophaga hutchinsonii*, Cy = *Cytophaga*, Sp = *Sporocytophaga*.

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CHAPTER 4

DYNAMICS OF SULFONOLIPIDS AND PHOSPHOLIPIDS DURING GROWTH OF *CYTOPHAGA HUTCHINSONII* ATCC# 33406 ON CELLULOSE POWDER IN STERILE SAND MEDIUM

Introduction

Gliding bacteria of the genera *Cytophaga* and *Flexibacter* contain an unusual sulfonolipid in their outer membrane (Godchaux and Leadbetter 1983) which may be a potential biomarker for these genera in soil. The constancy of sulfonolipids as a biomass component of *Cytophaga* and *Flexibacter* spp. during batch culture in liquid medium was reported in Chapter 3. Members of the genus *Cytophaga*, and less frequently of *Flexibacter*, are noted for their ability to hydrolyze complex substrates such as cellulose, chitin, starch, protein, agar, etc. (Christensen 1977). During growth on complex substrates (Stanier 1942) or when subjected to stress (Reichardt and Morita 1982), several cellulolytic and chitinolytic isolates form cells of aberrant morphology as the culture ages. These morphologies include nonrefractile coccoid cells, spindlelike and pestlelike rods, and rods with clear sacs attached at one end. The occurrence of coccoid cells with varying degrees of opacity under phase contrast microscopy has been likened to spheroplast formation (Leadbetter 1974), although ultrastructural studies have confirmed this only for *Cytophaga johnsonae* subjected to starvation (Reichardt and Morita 1982). The spheroplasts of *C. johnsonae* retain the membranous components of the cell envelope but appear to lack a murein sacculus (Reichardt and Morita 1982). With the exception of some coccoid cells of *C. johnsonae*, the viability of these aberrant cell forms is generally unknown, as is the fate of membrane phospholipids and sulfonolipids. The question then arises: can results from the growth of organisms under conditions of batch culture, where the cells are harvested and analyzed during exponential growth, be extrapolated to the behavior of sulfonolipids during prolonged growth of a *Cytophaga* sp. on a complex substrate?

My concern is not for the morphological and compositional changes within an isolate as it relates to cell ontogeny, but for the changes that may relate to the content, and hence the use of sulfonolipids as a biomarker for *Cytophaga* and *Flexibacter* spp. in soils.

The present chapter reports on the dynamics of sulfonolipid-S and phospholipid-P during growth of *Cytophaga hutchinsonii* on cellulose powder in sterile sand medium, a physical environment intermediate between liquid culture and soil.

Materials and Methods

Bacterial culture

C. hutchinsonii ATCC# 33406 was donated by Dr. R.G. von Tigerstrom, Department of Microbiology, University of Alberta. Inoculum for the culture flasks was prepared from log-phase cells of *C. hutchinsonii* grown in Dubos mineral medium (Dubos 1928) with 0.2 % glucose. An aliquot of cell culture was diluted 10-fold with saline-phosphate diluent, and 1 mL of diluted inoculum containing approximately 10^8 cells (1 nmol phospholipid-P) was added to each culture flask.

Culture flasks

Each culture flask culture was composed of 25 g 20 mesh Ottawa sand (heated overnight at 650 °C and washed with dilute acid and base) and 0.25 g cellulose powder (Whatman) in 125 mL Erlenmeyer flasks. The flask openings were closed with foam plugs (Dispo) and loosely capped with aluminum foil, then sterilized by autoclaving for 20 minutes on three consecutive days. Each flask received 6 mL of nutrient solution: 11.8 mM NaNO₃; 1.0 mM MgSO₄·7H₂O; 3.4 mM KCl; and 0.036 mM FeSO₄·7H₂O; 1 mL of 0.75% K₂HPO₄; and 1 mL of diluted bacterial culture. Radiolabelled ³⁵S-sulfate was added to each flask to give a specific activity of 1.16×10^6 dpm/μmol (19.3 kBq/μmol) sulfate.

The culture flasks were placed into 2 L Kerr jars containing 5 mL H₂O and the mouth of the jar sealed with Parafilm. For respiration measurements a glass jar containing 10 mL of 0.1 M NaOH was placed into four of the Kerr jars to trap evolved CO₂. The mouth of the Kerr jar was then covered with Parafilm and secured with a sealer lid. Lipids were extracted from four culture flasks and evolved CO₂ was measured on days 4, 7, 10, 13, 18, 24 and 30. On day 30, the culture flasks in the four Kerr jars used to measure evolved CO₂ were extracted for lipids. All culture flasks were incubated in the dark at 26 °C.

Phase-contrast photomicroscopy

Cell morphology was examined under phase-contrast using a Zeiss Universal microscope equipped with an Olympus OM2 camera containing Kodak TMax 100 black and white film.

Lipid extraction procedure

The lipid extraction procedure is a modification of the method of Bligh and Dyer as outlined in Kates (1986). Lipids were extracted by adding 48 mL solvent (methanol/chloroform, 2/1 Vol./Vol.) directly to the Erlenmeyer flasks containing *C. hutchinsonii* growing on cellulose powder in sand medium. The flasks were placed in a water bath and sonicated for 2 minutes, and incubated for 2 hours at room temperature with intermittent shaking. Aqueous ammonia was not included in the solvent (Godchaux and Leadbetter 1980) because N-acylcarnitines were the sole sulfonolipids present in *C. hutchinsonii* (Chapter 3), and preliminary trials with *C. hutchinsonii* in cellulose powder-sand culture verified the absence of carnitine. The crude lipid extract was filtered through Whatman #1 filter paper on a Buchner funnel. The sand-cellulose powder retained by the filter paper was then rinsed with an additional 30 mL solvent. The filtrate was transferred to a separatory funnel to which was added 25 mL chloroform and 32 mL 10 mM $(\text{NH}_4)_2\text{SO}_4$ and the phases allowed to separate overnight. Using higher molarity of $(\text{NH}_4)_2\text{SO}_4$ introduced excess water into the final chloroform extract, creating problems during subsequent evaporation steps. The chloroform phase was reduced to 5 mL by rotary evaporation, then filtered through Whatman #40 filter paper into a test tube. Benzene was added to the filtered chloroform extract during evaporation of the chloroform under nitrogen. The lipid residue was dissolved in 500 μL chloroform/methanol (1/1 Vol./Vol.), transferred to a glass vial and stored at -22°C .

A filtration procedure was used to determine whether membrane components were lost to the extracellular medium during conversion of rod-shaped cells to aberrant morphologies. The contents of an Erlenmeyer flask was gently filtered through Whatman #1 filter paper on a Buchner funnel. The sand-cellulose powder solids were then rinsed with diluted culture medium. To remove whole cells of *C. hutchinsonii* not retained by the filter paper, the filtrate was passed through a $0.2\ \mu\text{m}$ nylon filter (Millipore). The resulting filtrate was free of whole cells as determined by phase contrast microscopy. Solvent (methanol/chloroform 2/1 Vol./Vol.) was added to the filtrate to create a single phase and the lipids extracted as previously outlined.

All solvents were of high purity, glass distilled and filtered (Aldrich Chemical Company, HPLC grade). Chloroform was stabilized with amylenes.

Analytical methods

The following procedures were described in detail in Chapter 3: sulfonolipid assay by isotopic dilution of ^{35}S -labelled sulfate, phospholipid-P assay, and thin-layer chromatography and autoradiography of nonhydrolyzed lipid extracts. Evolved CO_2 was determined by potentiometric titration with 0.1 M HCl.

Results

After 4 days, a film of yellow growth was observed on the cellulose powder at the air-liquid interface, and numerous rod-shaped cells of *C. hutchinsonii* were present in the culture medium. Aberrant cells became apparent in the culture medium after 7 days incubation, and became more numerous during the course of the incubation (Figure 4.1).

Two, possibly three major ^{35}S -labelled sulfonolipids were detected by thin-layer chromatography and autoradiography (Figure 4.2). All exhibited the chromatographic behavior of N-acylcapsine as defined in Chapter 3. N-acylcapsines were the sole ^{35}S -labelled lipids present in *C. hutchinsonii* during the incubation period; the deacylated form, capsine, being absent.

The rate of CO_2 evolution peaked around 7 days, after which it declined then remained constant for the remainder of the incubation period (Figure 4.3). A total of 5-7 mg C was evolved as CO_2 over the 30 day incubation, or 6 % of added cellulose carbon.

The content of sulfonolipid-S continued to increase during most of the incubation, although the rate of increase declined gradually to zero between days 24 and 30 (Figure 4.4). For the first 13 days, phospholipid-P paralleled that of sulfonolipid-S; however, the content of phospholipid-P plateaued between days 13 and 24 before declining between days 24 and 30. The resulting phase shift in the dynamics of the two components gave rise to a steadily increasing ratio of sulfonolipid-S to phospholipid-P (Figure 4.5).

There did not appear to be a release of membrane components to the culture medium upon aging of the cells, because there was no detectable sulfonolipid-S or phospholipid-P in the extracellular medium after 30 days incubation (data not shown).

Discussion

Growth of *C. hutchinsonii* on cellulose powder in sterile sand medium produced aberrant growth forms similar in morphology to the pestlelike rods and coccoid cells described by Reichardt and Morita (1982) for *C. johnsonae*. The coccoid cells varied in appearance from dark to very light gray under phase contrast, the latter presumably moribund and devoid of cytoplasmic constituents. Aberrant cells were first observed in the sand-cellulose powder cultures of *C. hutchinsonii* after 7 days, after the period of maximum respiratory activity.

The growth dynamics of bacteria cultured in liquid medium under batch conditions follows a characteristic cycle known as the closed (or batch) culture growth cycle (Lynch and Poole 1979). In laboratory studies aimed at simulating natural environments many features of the growth cycle still apply (Lynch and Poole 1979). The dynamics of phospholipid-P during growth of *C. hutchinsonii* on cellulose powder appears to mimic the basic shape of the growth cycle, with the formation of aberrant cells beginning after the period of rapid accumulation of phospholipid-P (at 7 days). These aberrant cells are most likely unable to divide, and the relative proportions of these cells and cells still able to divide defines the retardation, stationary and death phases of the growth cycle (Drew, 1981). This conversion of rod-shaped cells to aberrant cells in cultures of *C. hutchinsonii* was not marked by a release of membrane components to the extracellular medium. Electron micrographs of *C. johnsonae* (Reichardt and Morita 1982) and a cellulolytic *Sporocytophaga* Sp13 (Chapter 1) reveal the presence of internal membranes (mesosomes?) in addition to cytoplasmic and outer membranes. The starved cells of *C. johnsonae* also contain these membranous structures (Reichardt and Morita 1982), but it is unclear whether all membrane components are preserved upon conversion of rod-shaped cells to coccoid forms. Thus, the decrease in phospholipid-P during the latter stages of the incubation may reflect catabolism of some phospholipids. In cultures of *C. hutchinsonii*, the onset of constant phospholipid-P was marked by a significant drop in respiratory activity which then held constant throughout the incubation.

The dynamics of sulfonolipid-S closely followed that of phospholipid-P for the first 13 days of incubation. During the stationary period of growth, as defined by a constant phospholipid-P content per culture flask, there was a steady increase in sulfonolipid-S followed by a plateau between days 24 and 30. Thus, either the catabolism of sulfonolipid-S occurred more slowly than phospholipid-P or the conversion to coccoid cells was marked by the synthesis of additional sulfonolipids resulting in a phase shift in the dynamics of the two components. It is unlikely that cells entering an unfavorable

growth period would expend energy to synthesize additional outer membrane components. A measure of cell protein would have helped in this regard, but the content of cellular protein in the cultures was too low to be measured accurately, and complete recovery of protein from the cells within the sand-cellulose powder cultures would be difficult.

The ratio of sulfonolipid-S to phospholipid-P of *C. hutchinsonii* growing on cellulose powder was not consistent between liquid- and sand-grown cells: 0.98 in liquid medium (Chapter 3) compared to 0.62 in sand medium at day 4. Furthermore, the ratio increased in sand-grown cells from 0.62 on day 4 to 1.2 on day 30. The source of this variation may lie in the content of phospholipid-P. First, in liquid grown cells of *C. hutchinsonii*, the content of phospholipid-P decreased while the content of sulfonolipid-S remained unchanged in response to a change in carbon source from glucose to cellulose powder (Chapter 3). Second, the content of sulfonolipid-S per gram wet mass of cells was similar between liquid- and surface-grown cells of *C. johnsonae* (Abbanat et al. 1988); however, they did not investigate the cellular content of phospholipid-P.

In light of the differences observed in the ratio of sulfonolipid-S to phospholipid-P between surface and liquid grown cells, and also between rod-shaped cells and aberrant cells, a hypothesis was formulated which would attribute these differences to changes in the content of internal membranes and therefore, phospholipid-P. Since sulfonolipids are allocated to the outer membrane (Godchaux and Leadbetter 1988), and sulfonolipids appear to be retained upon conversion of rod shaped cells to coccoid cells, I propose that the increase in the ratio of sulfonolipid-S to phospholipid-P observed upon conversion to coccoid cells reflects a loss of internal membranes. I also propose that surface grown cells contain additional internal membranes over liquid grown cells. To test this hypothesis, experiments on lipid composition would have to be conducted in conjunction with ultrastructural studies.

The potential use of sulfonolipids as a biomarker for viable *Cytophaga* and *Flexibacter* spp. in soils could be hampered if: (1) the aberrant cells formed in pure cultures of cellulolytic and chitinolytic cytophagas are prevalent in all *Cytophaga* and *Flexibacter* spp.; (2) these aberrant cells are moribund and not resting forms with the capacity for growth as postulated by Reichardt and Morita (1982); and (3) these aberrant cells persist in closed or bottle-neck type pores inaccessible to bacteriolytic organisms such as protozoa. These events would negate the use of sulfonolipids as a biomarker for viable cells in soil because total sulfonolipids would be a composite of sulfonolipids extracted from viable cells and from moribund cells protected from decomposition by virtue of their location in soil.

Conclusions

A clear difference in the dynamics of sulfonolipid-S and phospholipid-P was apparent in *C. hutchinsonii* while growing on cellulose powder in sterile sand medium. The dynamics of phospholipid-P closely matched the growth cycle as found in batch cultures. Sulfonolipid-S, however, accumulated in the cultures, either being metabolized more slowly than phospholipid-P or not metabolized at all. Thus, sulfonolipids do not accurately reflect the growth dynamics of *C. hutchinsonii* during monoculture on cellulose powder in sand medium. Further study is required to determine whether other isolates of *Cytophaga* and *Flexibacter*, particularly those less nutritionally specialized, also are incapable of metabolizing sulfonolipids in monocultures.

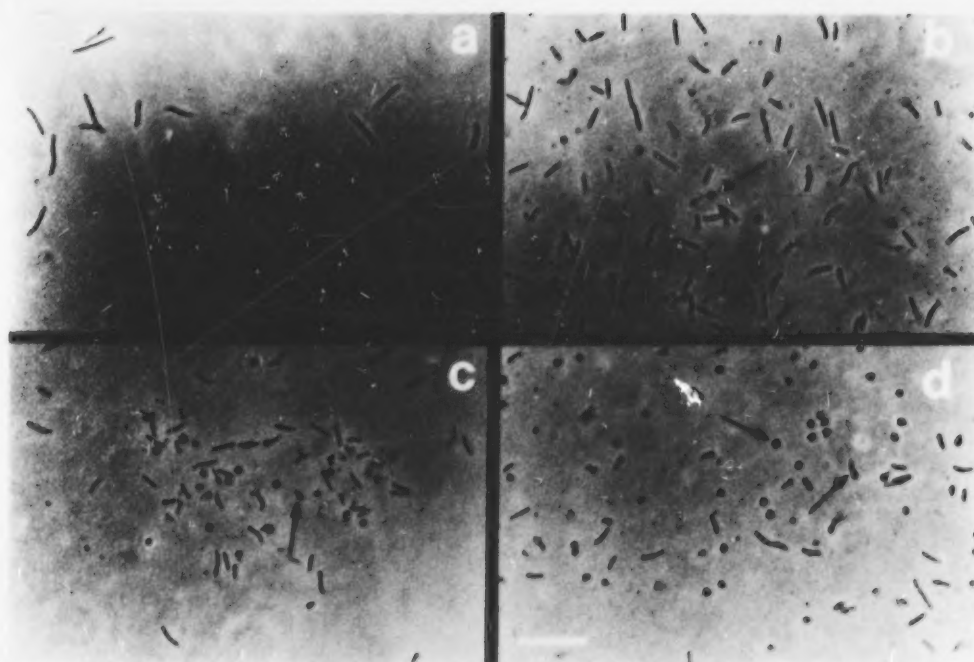


Figure 4.1. *Cytophaga hutchinsonii* growing on cellulose powder in sand medium at a = 4, b = 10, c = 18, and d = 30 days. Phase-contrast. Arrows: aberrant cells. Bars represent 10 μ m.



Figure 4.2. Autoradiogram of ^{35} S-labelled sulfonolipids of *Cytophaga hutchinsonii* separated by thin-layer chromatography. Lanes from left to right, days 4, 7, 10, 13, 18, 24 & 30. O = origin; Sf = solvent front.

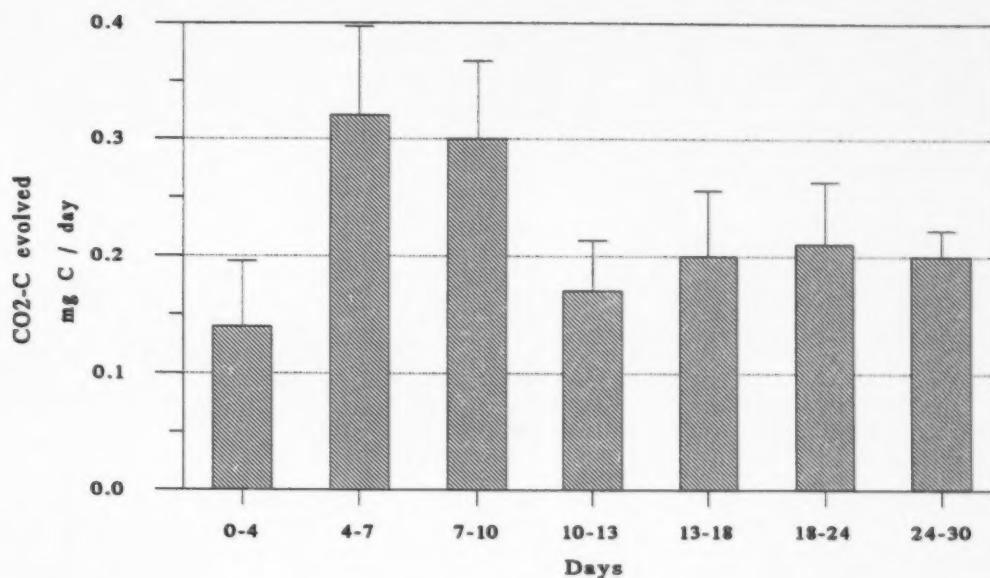


Figure 4.3. Rate of CO₂ evolution by *Cytophaga hutchinsonii* growing on cellulose powder in sand medium. Bars are sample standard deviations of the mean, n = 4.

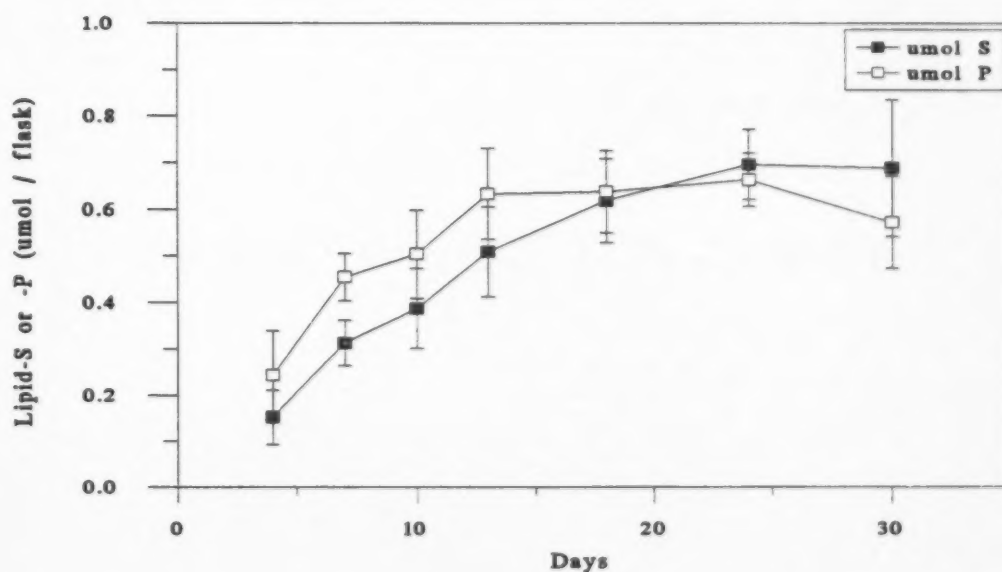


Figure 4.4. Sulfonolipid-S and phospholipid-P content of cultures of *Cytophaga hutchinsonii* growing on cellulose powder in sand medium. Bars are sample standard deviations of the mean, n = 4.

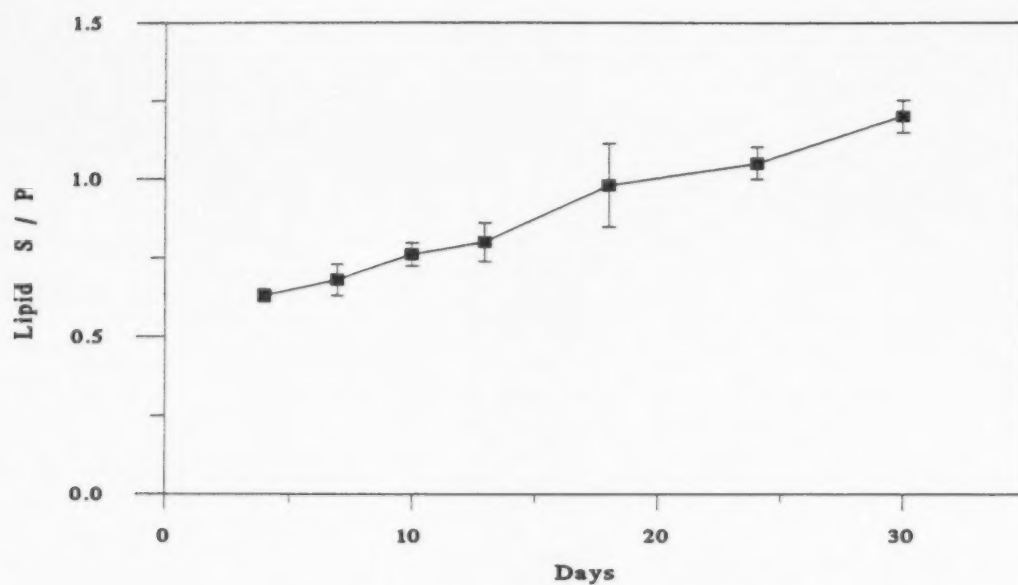


Figure 4.5. Changes in the ratio of sulfonolipid-S to phospholipid-P during growth of *Cytophaga hutchinsonii* on cellulose powder in sand medium. Bars are sample standard deviations of the mean, $n = 4$.

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CHAPTER 5

SULFONOLIPIDS AS A BIOMARKER TO MONITOR THE POPULATION DYNAMICS OF THE GENERA *CYTOPHAGA* AND *FLEXIBACTER* IN SOIL WORKED BY THE EARTHWORM *APORRECTODEA TURGIDA*¹

Introduction

The role of earthworms, particularly endogeic earthworms, in the reorganization of soil fabrics in the upper mineral horizons of grassland soils is well documented (Pawluk and Bal 1985). Endogeic earthworms live, feed and reproduce within the soil (Lavelle 1988). The highly dispersed nature of their food source, i.e. the soil organic matter and its associated microorganisms and fauna, requires them to ingest large quantities of soil material. For example, temperate earthworms ingest 1-2 times while tropical earthworms ingest 20-30 times their body mass daily (Lavelle et al. 1989). Most of the soil material passes through the earthworm gut and emerges as casts of molded excrement, called modexi.

There is evidence that earthworm casts are structurally, biochemically and microbiologically different from the soil material before ingestion. The selective ingestion of finer soil particles in combination with intimate mixing of the soil material during gut passage results in changes in pore size distribution, water holding capacity and aeration, thus creating new microhabitats for biota within the casts (Lee 1985). Enhanced mineralization and humification of soil organic matter during gut passage may alter the availability of substrates within the casts (Sharpley and Syers 1976; Barois and Lavelle 1986; Shaw and Pawluk 1986; Scheu 1987a; Edwards and Fletcher 1988; Lavelle 1988; Scheu and Wolters 1991). This in combination with changes in habitable pore space has been postulated to alter the composition and activity of the microbial community within the casts (Parle 1963; Loquet et al. 1977; Shaw and Pawluk 1986; Scheu 1987b; Wolters and Joergensen 1992).

Of the several approaches used to monitor microbial biomass in natural habitats, i.e. cultural, microscopic, metabolic, biochemical and genetic (Karl 1986; Sayler et al. 1992),

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lipid biomarkers may provide a range in specificity unobtainable by any other method. In their study of microbial consortia in sediments, D. C. White and coworkers have used lipid biomarkers not only to determine total biomass of sediments, but also to elucidate community structure from profiles of phospholipid-linked fatty acid methyl esters (PL-FAMES) in addition to 'signature' lipids specific to particular subsets of the population (White et al. 1979b; White 1983; White 1986). In a recent review of biochemical methods for the analysis of microbial communities in soil, however, the limited application lipid biomarkers have received was immediately apparent (Tunlid and White 1992).

The presence of a unique sulfonate-containing lipid in the outer membrane of gliding bacteria belonging to the genera *Cytophaga* and *Flexibacter* led to the examination of sulfonolipids as a potential biomarker for these organisms in soil. Collectively, these sulfonolipids are termed capnoids, the most common member being the N-acylated version, N-acylcapnine (Godchaux and Leadbetter 1983). Earlier I reported on the constancy of sulfonolipids in several isolates of *Cytophaga* and *Flexibacter* subjected to differing growth conditions in batch culture (Chapter 3). Here I complete my evaluation of sulfonolipids as a biomarker for the genera *Cytophaga* and *Flexibacter* in soil, then apply the technique together with analysis of PL-FAMES to soil influenced by the activities of an endogeic earthworm, *Aporrectodea turgida*.

Specific questions are:

1. Can sulfonolipids, when added within whole cells, be recovered quantitatively from soil?
2. Do sulfonolipids, when added within whole cells, persist in soil in an extractable form upon cell death?
3. Does the addition of the earthworm *Aporrectodea turgida* to soil microcosms influence the community structure and dynamics of the soil population as determined by phospholipid-P and PL-FAMES?
4. Does the addition of the earthworm *Aporrectodea turgida* to soil microcosms influence the dynamics of *Cytophaga* and *Flexibacter* spp. as determined by sulfonolipids?

Materials and Methods

Soil and earthworms

Soil was sampled from the Ap horizon of zero tillage plots located at the Ellerslie Research Station (NE24-51-25-W4), 10 km south of Edmonton, Alberta. The soil is an Eluviated Black Chernozemic (silty clay loam texture, 6.5 % C, 0.53 % N, pH 6.1) (Bowser et al. 1962), and has been cropped to barley (*Hordeum vulgare* (L.) cv. Empress) since 1979. The soil was passed through a 2 mm sieve, then stored moist at 4 °C. All subsequent referrals to soil are on an oven-dry mass basis. Partially decomposed barley straw was collected from the site, dried at room temperature, then ground to 0.5 mm in a Wiley mill.

Adult earthworms were collected by hand from the same site as the soil sample, placed into soil at 10 °C and fed regularly with barley straw. The worms were classified as *Aporrectodea turgida* (Eisen) (Reynolds 1977).

Recovery and persistence of sulfonolipids added within whole cells to soil

Cytophaga hutchinsonii (ATCC# 33406) was batch cultured in 1L Erlenmeyer flasks with 200 mL of NG medium (Chapter 3) containing ^{35}S -sulfate to a specific activity of 4.36×10^5 dpm/ $\mu\text{mol S}$ (7.27 kBq/ $\mu\text{mol S}$). The cells were harvested in late log phase by centrifuging at 4 °C and 10,000 $\times g$ for 15 minutes. The cell pellet was washed once with diluent (0.85 % NaCl, 0.03 % KH_2PO_4 , 0.06 % Na_2HPO_4) and once with water. Water was added to the cell pellet to form a homogeneous slurry of cells. A 0.5 mL aliquot of cell slurry was transferred to each of three Teflon centrifuge tubes and the lipids extracted as previously described, except $\text{NH}_3(\text{aq})$ was omitted from solvent I (Chapter 3). Each 0.5 mL of cell slurry contained 1.12 $\mu\text{mol S}$ as sulfonolipids.

Ten grams of soil was weighed into 50 mL Erlenmeyer flasks and moistened to a gravimetric water content of 0.42 kg H_2O /kg soil. The soil was incubated for 10 days at 22 °C, after which time 0.5 mL of cell slurry of *C. hutchinsonii* was mixed into the soil in each flask. Three flasks were extracted for ^{35}S -labelled sulfonolipids by the method outlined below within 20 minutes of the initial mixing of the cells into the soil. The remaining flasks were placed into 2 L Kerr jars containing 5 mL H_2O . Parafilm was applied to the mouth of the Kerr jar to prevent moisture loss and allow gaseous exchange,

and the jars were incubated at 22 °C. Four replicates were extracted for ^{35}S -labelled sulfonolipids on days 10, 21 and 29.

Community dynamics in soil worked by *Aporrectodea turgida*

Preparation of microcosms

Sieved, moist soil was mixed with barley straw to a final concentration of 0.05 % straw. Microcosms were prepared by weighing 30 or 60 g portions of soil + straw into plastic containers (300 mL). A solution of K_2SO_4 was added to the soil at a rate of 2.5 $\mu\text{mol S/g soil}$ (80 $\mu\text{g S/g soil}$) to bring the gravimetric moisture content to 0.42 kg $\text{H}_2\text{O/kg soil}$. For radiolabelled soil, ^{35}S -sulfate was added to the K_2SO_4 solution at a specific activity of $9.6 \times 10^6 \text{ dpm}/\mu\text{mol}$ (160.6 kBq/ μmol) sulfate, or $2.4 \times 10^7 \text{ dpm/g soil}$ (401.7 kBq/g soil). As sulfate concentrations were 8 times the concentration in the soil sample, a high specific activity of sulfate was necessary to obtain measurable counts of ^{35}S -labelled sulfonolipids. The plastic containers were sealed with vented lids and placed into plastic bags containing 5 mL H_2O . After 3 days of incubation at 18 °C, the soil was stirred to distribute the added sulfate evenly, and then left undisturbed for a further 9 days for respiration measurements, or for a further 14 days for lipid extraction and extractable sulfate measurements. Because the preincubation periods differed between the respiration and lipid extraction experiments, the dates after addition of the earthworms are not directly comparable but are staggered by 5 days.

Respiration

After 12 days of incubation, two earthworms were added to each of six microcosms containing 30 g soil; two of the microcosms had soil labelled with ^{35}S -sulfate. Check treatments consisted of identical microcosms without earthworms. The soil containers were placed into 2 L Kerr jars containing 5 mL H_2O and a jar with 10-20 mL 0.1 M NaOH to trap evolved CO_2 . The mouth of the Kerr jar was then sealed with Parafilm and a sealer lid, and secured with a metal band. The worms were removed after 5 days incubation, and evolved CO_2 was measured on days 5, 10, 15, 25, 35, 45, and 55 by potentiometric titration with 0.1 M HCl. The results presented are means of four replicates from nonradiolabelled soil.

Soil labelled with ^{35}S -sulfate

After 17 days of incubation, four earthworms were added to each of three microcosms containing 60 g soil labelled with ^{35}S -sulfate. The number of worms per 60 g soil was increased to six or eight in microcosms where the worms were removed after 1 or 3 days, respectively. The worms from the remaining microcosms were removed after 5 days. The soil was then extracted for lipids and ^{35}S -sulfate on days 1, 5, 10, 15, 20, 30, 40 and 50. Check treatments consisted of microcosms containing 30 g soil and without earthworms.

Nonradioactive soil

Microcosms were prepared as for the radiolabelled soil but without the radioactive ^{35}S -sulfate. Soil from the worm-worked and check treatments was extracted for total sulfate on days 5, 10, 15, 20, 30, 40 and 50. Earthworm fecal pellets were removed from the worm-worked soil on days 1, 15, 30 and 50, and photographed under a dissecting microscope and by scanning electron microscopy.

Analytical methods

Specific activity of sulfate in the soil

The specific activity of sulfate in the soil was monitored during the incubation period by extracting the soil with 0.01 M CaCl_2 (Tabatabai 1982). Total radioactivity was determined from extracts of soil labelled with ^{35}S -sulfate. A 0.5 mL aliquot of soil extract was transferred to a 20 mL scintillation vial and 10 mL of Scintiverse II (Fisher Chemical Company) was added. Disintegrations per minute were determined by efficiency tracing (Ishikawa et al. 1984) on a Packard 2000A Liquid Scintillation Analyzer. An automated colorimetric assay based on complexation of sulfate with methylthymol blue (Technicon autoanalyzer, Alberta Agriculture Soils and Animal Nutrition Laboratory) was used to measure total sulfate in extracts of nonradiolabelled soil.

Lipid extraction of soil labelled with ^{35}S -sulfate

Before sampling, the soil from each microcosm was gently stirred to distribute the earthworm fecal pellets evenly. A 20 g sample of soil was transferred to a glass mortar and 8 mL of 50 mM K_2HPO_4 buffer (pH = 7.4) and approximately 10 mL solvent (methanol/chloroform, 2/1 Vol./Vol.) was added to the soil. The soil aggregates were gently crushed with a pestle and the resulting soil slurry transferred to a 125 mL Erlenmeyer flask with 96 mL solvent. The flasks were placed in a water bath and sonicated for 2 minutes, then allowed to sit for 2 hours at room temperature with intermittent shaking. The crude lipid extract was filtered through Whatman #1 filter paper on a Buchner funnel. The soil solids on the filter paper were then rinsed with 30 mL solvent. The filtrate was transferred to a 250 mL separatory funnel to which was added 38 mL chloroform and 48 mL 10 mM $(\text{NH}_4)_2\text{SO}_4$ and processed as previously outlined (Chapter 4). The lipid residue was quantitatively transferred to a 2 mL glass vial, the solvent evaporated under nitrogen, and the residue redissolved in 1.0 mL chloroform/methanol (1/1 Vol./Vol.). The lipid extracts were stored at -22°C .

All solvents were of high purity, glass distilled and filtered (Aldrich Chemical Company, HPLC grade). Chloroform was stabilized with amylenes.

Phospholipid and sulfonolipid assays

Phospholipid-P was determined as described in Chapter 3. The content of ^{35}S -labelled sulfonolipids was determined from the radioactivity in the sulfonolipids and the specific activity of the sulfate solution added to the soil. The presence of ^{35}S -labelled lipids other than sulfonolipids in the lipid extracts was determined by thin-layer chromatography and autoradiography (Chapter 3). The interfering lipids were removed from a 100 μL aliquot of lipid extract by a combination of mild alkaline hydrolysis (White et al. 1979c) and acid hydrolysis (Godchaux and Leadbetter 1983). After acid hydrolysis, aqueous ammonia was added to the extract to deprotonate the free amino group of capnine, and the sulfonolipids were collected in the chloroform phase upon addition of chloroform and water. The ^{35}S -sulfate present in the lipid extracts as ester sulfate or inorganic sulfate was removed with the aqueous phase. The ^{35}S -labelled sulfonolipids in the chloroform phase were transferred to a 20 mL scintillation vial, the chloroform evaporated, and the residue dissolved in 0.5 mL 0.1 % sodium dodecyl sulfate and 10 mL Scintiverse II (Fisher Chemical Company). Disintegrations per minute were

determined by efficiency tracing (Ishikawa et al. 1984) on a Packard 2000A Liquid Scintillation Analyzer.

Phospholipid-linked fatty acid methyl esters

Phospholipids were separated from ^{35}S -labelled lipid extracts collected on day 1, 10, 30 and 50 by column chromatography with silica gel as the adsorbent. A 600 μL aliquot of lipid extract was applied to the top of a 10 x 30 mm chromatography column containing 1.5 g of silica gel (Davisil, 100-200 mesh, Aldrich Chemical Company) in chloroform. The neutral lipids and glycolipids were eluted from the silica gel with 10 mL chloroform and 10 mL acetone, respectively. The polar lipids (mainly phospholipids and ^{35}S -labelled sulfonolipids) were then eluted with 10 mL methanol into a 15 mL Pyrex test tube with a Teflon-lined screw cap. The methanol was evaporated under N_2 at 37 °C.

The phospholipids were hydrolyzed and the resulting fatty acids methylated by mild alkaline hydrolysis (White et al. 1979c). This procedure does not hydrolyze the N-acyl group from sulfonolipids as verified by thin-layer chromatography and autoradiography of ^{35}S -sulfonolipids and the results of Godchaux and Leadbetter (1980). The resulting fatty-acid methyl esters (FAMES) were isolated in the chloroform phase, the solvent reduced in volume under N_2 , then passed through a second chromatography column containing silica gel to remove contaminating ^{35}S -labelled sulfonolipids and other polar components. The FAMES were eluted from the silica gel into a Pyrex test tube with 10 mL chloroform, the chloroform evaporated under N_2 , and then were quantitatively transferred to a 2 mL vial. The solvent was removed under N_2 and the FAMES redissolved into 50 μL hexane containing the internal standard methyl-nonadecanoate.

The FAMES were separated by capillary gas chromatography on a Hewlett Packard 5790A gas chromatograph in split mode (50:1) housing a fused silica capillary column with a nonpolar bonded phase (30 m, 0.25 mm I.D., 0.1 μm film thickness, Rtx-1 Restek Corporation). Helium was used as the carrier gas with a flow rate of 25 cm sec^{-1} . The oven temperature was held at 100 °C for 1.0 min. then increased at 2.5 °C min^{-1} to 225 °C. The injector and flame ionization detector temperatures were 250 °C and 280 °C, respectively. An internal standard, methyl-nonadecanoate (0.4 $\mu\text{g}/\mu\text{L}$) was added to the FAME extract to ensure reproducibility in the amount of lipid entering the capillary column. Identification of the FAMES was by comparison of retention time and equivalent chain length with known standards (Bacterial Acid Methyl Esters CP Mix,

Supelco Canada Ltd.). The relative elution order and the identity of each standard was confirmed by gas chromatography mass spectrometry. Individual FAMES were reported as ratios of peak areas to methyl-hexadecanoate (palmitic acid), the most abundant FAME in the sample, which has a retention time of 25.5 minutes.

Fatty acid nomenclature

The shorthand designation for fatty acids as used in Figures 5.6 and 5.7 gives the total number of carbon atoms (inclusive of methyl branches) before the colon and the number of double bonds after the colon. Methyl branching is denoted by the prefixes 'i' for iso, where the methyl group is located on the second to last carbon of the chain, and 'a' for antiso, where the methyl group is located on the third carbon from the end of the chain. The position of double bonds is given in brackets immediately after the numeral indicating the number of double bonds, and the location is given in reference to the carboxyl carbon, assigned C1. The position of hydroxyl groups is also located in reference to the carboxyl carbon.

Statistical analysis

The PL-FAMES were clustered by a single linkage method with distances given by Pearson's correlations (SYSTAT: Wilkinson 1986).

Microscopy of earthworm fecal pellets

Photomicroscopy and scanning electron microscopy

Earthworm fecal pellets were removed from nonradiolabelled soil microcosms on days 1, 15, 30 and 50, and photographed under a Wild stereomicroscope using Kodak color film, ASA 100. In preparation for SEM, fecal pellets were gently placed into porous quartz holders with small squares of Kimwipes separating individual modexi. The fecal pellets were fixed overnight in 2.5 % glutaraldehyde in phosphate buffer, pH 7.2, washed twice in phosphate buffer, then dehydrated for 3-4 hours each in a graded series of ethanol in water: 30, 40, 50, 60, 70, 80, 90 and 100 %. The fully dehydrated fecal pellets were then critical point dried with liquid CO₂, coated with gold in a sputter coater, and examined by a Cambridge Stereoscan 250 scanning electron microscope.

Results

Recovery and persistence of ^{35}S -labelled sulfonolipids added to soil

Sulfonolipids, when added to soil within whole cells, were quantitatively recovered (98 %) by the lipid extraction procedure (Figure 5.1). Within 10 days of adding ^{35}S -labelled cells of *C. hutchinsonii* to soil, only 25 % of the ^{35}S -labelled sulfonolipids were recovered and by 29 days this percentage had decreased to 10 %. A single component first order decomposition model:

$$\ln (A_t / A_0) = -0.238 - 0.081 t \quad [1]$$

where A_0 = amount of sulfonolipid added to soil and A_t = amount of sulfonolipid remaining at time = t (days), accounted for 94% of the variation in the data, although the y-intercept was considerably less (78.8 %) than the measured 98 % recovery of sulfonolipids from cells added to soil.

Microbial dynamics and activity in soil worked by *Aporrectodea turgida*

Earthworm activity

Active burrowing and cast production was evident in all soils at days 1, 3 and 5. Upon removal of the earthworms on day 5, approximately 20-25 % of the soil material had been transformed into casts. Most of the casts existed as molded excrement (modexi) of coalesced smaller ellipsoid units (Figure 5.3). Surface colonization of the casts by fungal hyphae was evident in as little as 3 days after excretion, and became more abundant as the casts aged.

The smooth surface of the casts was especially apparent by scanning electron microscopy (Figure 5.4a). Shrinkage cracks were numerous, but it is unclear if these occurred during fixation and dehydration of the casts for analysis or if they are a feature caused by drying of the casts in the soil. A close-up view of the cast surface showed abundant mucus, possibly mixed with clay (Figure 5.4b). Two types of mucus were evident, one consisting of flattened sheets (Figure 5.4b) the other more fibrous (Figure 5.4c). The absence of a smooth fracture surface (Figure 5.4d) suggested the interior of the casts was a heterogeneous collection of juxtaposed elements creating numerous

microhabitats of varying sizes. Fungal hyphae were often observed on the surfaces of the casts, and in some instances hyphae were heavily coated with material which would suggest passage through the earthworm gut. Bacteria, however, were not observed either in the interior of the casts or on the surface, but their outlines may have been obscured by coatings of clay and mucus.

Rate of CO₂ evolution

The presence of earthworms significantly increased respiration from the worm-worked soil for the first 5 days, presumably due in part to the worms' own respiration (Figure 5.2). Removal of the worms on day 5 significantly reduced respiration, but the effects of the worms on the indigenous microflora and microfauna was still apparent until 15 days. After 15 days of incubation the stimulatory effect of the worms was exhausted, and there was no difference in respiration rate between the worm-worked and the check soils.

The dynamics of CO₂ evolution in the check soil and the soil worked by the earthworm *Aporrectodea turgida* was biphasic. Despite a preincubation period of 12 days, the rate of CO₂ evolution in the check soil had not stabilized. In fact, the respiratory rate declined sharply from days 0 to 15, then increased significantly between days 15 and 25 before declining gradually between days 25 and 55.

Respiration rate from both check and worm-worked soil microcosms containing radiolabelled ³⁵S-sulfate was not significantly different ($p=0.05$) from nonradiolabelled soil microcosms.

Dynamics of phospholipid-P and FAMES

Extractable phospholipid-P in both the check and worm-worked soils ranged between 70 and 80 nmol P/g soil throughout the incubation (Figure 5.5). Addition of worms to the soil resulted in a slight reduction in phospholipid-P between days 1 and 5 while in the check soil phospholipid-P increased slightly during this same period. During the remainder of the incubation, the dynamics of phospholipid-P in the worm-worked soil closely paralleled that of the check soil, although the gap between the two curves widened between days 20 and 40.

The ratio of palmitic acid (C16:0), the most abundant fatty acid in all samples, to the internal standard methyl-nonadecanoate (C19:0), was consistent throughout the

incubation for both the check and worm-worked soils (Figure 5.6), thus following the dynamics of phospholipid-P (Figure 5.5). Cluster analysis of the PL-FAME profiles failed to distinguish between the check and worm-worked soils, and among dates (Appendix C). The distribution of PL-FAMES in the phospholipid fraction of lipids extracted from the check soil 1 day after the preincubation period of 17 days (Figure 5.7) was typical of both the worm-worked and check soils throughout the incubation.

Dynamics of ^{35}S -labelled sulfonolipids

There was no change in extractable sulfate or radioactivity during the incubation period; hence, the specific activity of sulfate in the soil could be used with confidence to calculate the content of ^{35}S -labelled sulfonolipids by isotope dilution. Only a small percentage ($< 0.04\%$) of the ^{35}S -label added to the soil was recovered in the lipid fraction. Of this percentage, approximately 75 % of the ^{35}S -label remained at the origin of the TLC plate, 10 % was located in zones corresponding to sulfonolipids (Chapter 3), and the remaining 15 % formed a smear extending from the origin to the solvent front of the TLC plate (Figure 5.9).

After the preincubation period of 17 days, ^{35}S -labelled sulfonolipids were easily detected in the acid/base hydrolyzed lipid extracts. The efficiency of the acid/base hydrolysis procedure in removing contaminating ^{35}S -sulfate and ^{35}S -labelled lipids other than sulfonolipids was indicated by the presence of capnine as the sole product in the hydrolyzed lipids upon TLC and autoradiography (Figure 5.9). Recoveries of N-acylcapnine extracted from *C. hutchinsonii*, and taken through the hydrolysis procedure were greater than 90 %. The radioactivity in capnine was equivalent to the amount of N-acylcapnine in the nonhydrolyzed extract; hence, the smearing of ^{35}S -lipids noted upon TLC/autoradiography was not due to adsorption of N-acylcapnine to other lipid components which would affect its migration on the TLC plate.

In the check soil a net increase in ^{35}S -labelled sulfonolipids was observed between days 1 and 30, followed by a slight decline between days 30 and 40 (Figure 5.8). Between days 40 and 50 there was no change in the content of ^{35}S -labelled sulfonolipids. Addition of earthworms to the soil altered the dynamics of ^{35}S -sulfonolipids between days 1 and 15, and then again at days 30 to 50. The dynamics of ^{35}S -labelled sulfonolipids in the worm-worked soil closely paralleled that of the check soil between days 15 and 30.

Discussion

Recovery and persistence of ^{35}S -labelled sulfonolipids added within whole cells to soil

The recovery of sulfonolipids from cells of *Cytophaga* and *Flexibacter* naturally occurring in soil is dependent upon (1) the accessibility of the cells within the soil to the extracting solvent; (2) the efficiency of the extracting solvent to solubilize sulfonolipids from the cells; (3) the partitioning of the sulfonolipids between the extracting solvent and the soil matrix; and, (4) losses of sulfonolipids during subsequent manipulations. Previously it was shown that sulfonolipids were quantitatively recovered from cells of *C. hutchinsonii* by the extracting solvent and that losses of sulfonolipids during the extraction procedure totaled 1 % (Chapter 3). The accessibility of cells growing in soil to the extracting solvent cannot be evaluated; however, the recovery of sulfonolipids from ^{35}S -labelled cells of *C. hutchinsonii* added to soil was nearly complete (98 %, sd = 3 %, n = 3), suggesting complete partitioning of the ^{35}S -labelled sulfonolipids into the extracting solvent with no adsorption of sulfonolipids onto the soil matrix.

The recovery of lipid components added to soils is highly dependent on whether the lipid exists as an isolated compound or is cell-bound, and the physical state of the soil (i.e. moist or lyophilized). When ergosterol is added to soil as fungal mycelia recoveries of 96 % have been obtained (Davis and Lamar 1992). Addition of pure ergosterol to soil in solvent results in recoveries ranging from 76 to 96 % (Davis and Lamar 1992; Grant and West 1986). Similar recoveries were obtained for ^{14}C -labelled phospholipids extracted from *E. coli* and added in chloroform to lyophilized mud (White et al. 1979c). Lyophilization of the soil after amendment with ergosterol (Johnson 1989) or mycelia (Davis and Lamar 1992) reduced recoveries of ergosterol; however, losses from mycelia could be prevented by rapidly freezing the samples to prevent fungal autolysis during lyophilization (Davis and Lamar 1992).

The addition of bacterial cells to nonsterile soil is often marked by a 10- to 100-fold reduction in the number of culturable cells within a few days (Acea et al. 1988; Thompson et al. 1990). Rates of mineralization of ^{14}C -, ^{15}N - and ^{32}P -labelled cells (van Veen et al. 1987) and ^{14}C -labelled cell components (Nelson et al. 1979) added to soil are attributed to a rapid death and extensive decomposition of the added cells in addition to conversion to viable but nonculturable cells. The reduction in extractable ^{35}S -labelled sulfonolipids from whole cells of *C. hutchinsonii* added to soil indicates a rapid decomposition of the added cells with a concomitant degradation or adsorption to soil of their sulfonolipid components. The accessibility to microbial attack of ^{14}C -labelled

phosphatidyl choline adsorbed onto sand then mixed into nonsterile soil (Tollefson and McKercher 1983) suggests that even adsorbed sulfonolipids may be degraded. The first order decay constant for sulfonolipids, $k=0.081 \text{ days}^{-1}$, is smaller than the average decay constant for the labile fraction of cell walls added to soil, $k=1.6 \text{ week}^{-1}$ or 0.23 day^{-1} (Juma and McGill 1986) and may reflect the addition of viable cells to soil rather than lyophilized cells and cell fractions (Nelson et al. 1979). Thus, sulfonolipids do not persist in an extractable form in soil once the cells are degraded.

Microbial dynamics and activity in soil worked by *Aporrectodea turgida*

The respiratory behavior of the check soil was unusual in two respects: (1) despite a preincubation period of 12 days, respiration in the check soil had not stabilized by the beginning of the experiment, but continued to decline for a further 15 days; and, (2) a flush in CO_2 occurred between 15 and 20 days after which the rate of CO_2 evolution appeared to stabilize. A further characteristic is that the brief presence of earthworms in the soil during days 1 to 5 changed the magnitude but not the pattern of CO_2 evolution. Thus, the pattern of respiration was a feature of the soil as dictated by the incubation conditions and the presence of straw.

The flush of CO_2 due to manipulation of the soil generally subsides within 10 days of incubation (Shan-Min et al. 1987). The constant flux of CO_2 from soil after this period is termed "basal respiration" and appears to be related to the availability of carbon and not to the size of the microbial biomass (Shan-Min et al. 1987). In laboratory incubations, the addition of 2 % w/w straw to soil delayed the onset and increased the magnitude of basal respiration when compared to nonamended soil (Ocio and Brookes 1990).

Lipid phosphate as a measure of total biomass in sediments dominated by prokaryotes has been shown to satisfy the criteria outlined by Jenkinson and Ladd (1981) for a successful biomarker: the phospholipid-P content of 13 diverse bacterial monocultures subjected to a variety of environmental stresses fell within the range 10-120 $\mu\text{mol-P/g}$ dry mass of cells (tabulated by White et al. 1979a); recovery of ^{14}C -labelled lipids from *E. coli* added to lyophilized sediments was quantitative (White et al. 1979c); extractable phospholipid-P correlates well with other measures of biomass in subsurface sediments: extractable ATP, rate of DNA synthesis, and direct counts (White et al. 1979c; Balkwill et al. 1988); and phospholipids are actively metabolized in bacterial monocultures (White and Tucker 1969) as well as in sediments (White et al. 1979c).

The use of phospholipid-P as a measure of total biomass in soils is limited (Tunlid and White 1992) and there is only one report where it has been compared, albeit unfavorably, to other measures of total biomass (Zelles et al. 1992). A high correlation was found among 8 agricultural soils between total FAMES and several measures of microbial biomass ($r=0.896-0.967$) as well as with palmitic acid ($r=0.979$) (Zelles et al. 1992). A poor correlation was found with lipid phosphate, although, their reported lipid phosphate values were only 1/10th of expected values based on total FAMES and assuming 2 fatty acids per phospholipid. Similar discrepancies were found upon conversion of biomass C to lipid phosphate using factors of 50 $\mu\text{mol P/g}$ dry mass of cells (White et al. 1979a) and 0.45 g C/g dry mass of cells (Paul and Clark 1989).

Limitations to the technique in soil stem from incomplete knowledge of the phospholipid concentrations in the various soil biota under differing growth conditions and morphological states (i.e. spores, cysts, resting cells, etc.). With the latter, two further concerns arise: (1) the proportion of the soil biota present in resting forms, and (2) the efficiency of extraction of phospholipids from these forms.

By combining lipid phosphate with analysis of FAMES some of the uncertainties surrounding lipid phosphate as a measure of biomass can be resolved for the present study. Manipulation of natural microbial communities in the laboratory to select for certain groups of organisms was monitored successfully by changes in profiles of phosphoester-linked fatty acids (Bobbie and White 1980; Parkes and Taylor 1985; Federle et al. 1983; Taylor and Parkes 1985; Tunlid et al. 1985). The sensitivity of FAME profiles to the growth stage and environment of the organism (Rose 1989) necessitates caution when attributing changes in FAME profiles to changes in species composition. The similar distribution of FAMES between the worm-worked and check soils throughout the duration of the experiment suggested little change in community composition or physiological state of the majority of the soil biota. Thus, either most of the biota were quiescent or the relative proportions of the various taxa remained unchanged during turnover.

Because phospholipid-P, ratios of palmitic acid to methyl-nonadecanoate (internal standard), and FAME profiles showed little variation over the incubation period, lipid phosphate can be used as a measure of the soil biomass under the present conditions. Total biomass, as measured by lipid phosphate, changed little during the course of the incubation although the dynamics of the means bore a superficial resemblance to CO_2 evolution, particularly in the check soil. The dates given on the x-axes are not directly comparable since the preincubation periods differed between the two experiments: phospholipid-P at 10 days corresponds to CO_2 evolved between 10-15 days. Thus, the

net decrease in biomass between days 5 and 15 followed by the accumulation of biomass between days 15 and 20 may account for the flush of CO₂ which occurred between days 15 and 25. Using conversion factors of 50 µmol P/g dry mass of cells (White et al. 1979a), 0.45 g C/g dry mass of cells (Paul and Clark 1989) and a carbon utilization efficiency of 40% (van Veen et al. 1985) a decrease in biomass corresponding to 5 nmol-P/g soil could release 27 µg CO₂-C/g soil if it were consumed by surviving populations. A gross flux of C as CO₂ through the biomass 2 times greater than that attributable to the net change in biomass could easily account for the flush of CO₂ (51.7 µg CO₂-C/g soil) over the 15 to 25 day period.

In this study, microbial biomass was not determined by the chloroform-fumigation incubation method due to the addition of straw to the soil (Ocio and Brookes 1990). Using the above conversion factors, total biomass as given by phospholipid-P was equivalent to 625-734 µg C/g soil. This range in biomass is greater than the 470-590 µg C/g soil reported for soil sampled directly from neighbouring field plots and analyzed by chloroform-fumigation incubation (Rutherford and Juma, 1989; Dinwoodie and Juma 1988).

The idea of increased biomass upon passage of the soil through geophagic earthworms is not supported by my results nor is it supported by other researchers when biomass was determined by noncultural methods, such as substrate-induced respiration (Scheu 1987b) and chloroform-fumigation extraction (Daniel and Anderson 1992). In contrast, a significant reduction in microbial biomass was found in 5 out of 6 soils under beech forest that were continuously worked for 21 days by the endogeic earthworm *Aporrectodea caliginosa* (1 worm per 70 g soil) (Wolters and Joergensen 1992).

The similarity in PL-FAME profiles between worm-worked (20-25 % casts) and check soils is interpreted as evidence that the soil microbial community structure is not measurably altered upon passage through the earthworm gut. Small changes in the active microflora would have been masked by the much larger and inactive biomass present in the soil and casts. Active microbial biomass accounted for 5.0 % of the total biomass in 4 day old casts and 4.5 % of the total biomass in soil as measured by oxygen consumption following glucose addition (Scheu 1992). Recent investigations on the ultrastructure of soil passed through the earthworm gut supports passage of intact microaggregates up to 200 µm in diameter which are not settled with larger, and presumably recently active bacteria (Altemüller and Joschko 1992; Lee and Foster 1991). These latter cells are concentrated in streaks of high slime content between soil material and in voids (Altemüller and Joschko 1992).

The presence of ^{35}S -labelled sulfonolipids in lipid extracts from both the check and worm-worked soils 18 days after the introduction of ^{35}S -sulfate confirmed the growth of *Cytophaga* and *Flexibacter* spp. in soil. If the equation:

$$y = 0.137 + 0.00797 t - 0.00011 t^2 \quad r = 0.9996 \quad [2]$$

describing net accumulation of ^{35}S -sulfonolipids between days 1 and 30 in the check soil is extrapolated to $y = 0$, ^{35}S -labelled sulfonolipids would have first appeared 3 days after addition of ^{35}S -sulfate to the soil.

Eighteen days after addition of ^{35}S -sulfate to the soil, the ^{35}S -labelled population of *Cytophaga* and *Flexibacter* contained approximately 0.34 nmol phospholipid-P based on a ratio of sulfonolipid-S to phospholipid-P of 0.4 (Chapter 3), or 0.44 % of the total phospholipid-P in the biomass. By day 50, the ^{35}S -labelled population of *Cytophaga* and *Flexibacter* represented 0.9 to 1.2 % of the total biomass. The ability of some species of *Cytophaga* and *Flexibacter* to incorporate sulfur from protein while in the presence of abundant inorganic sulfate (Chapter 3) implies that sulfonolipid contents based on the specific activity of sulfate in the soil may be minimum estimates.

Attempts were made to measure the total content of sulfonolipids in the soil, which would include sulfonolipids in the quiescent population of *Cytophaga* and *Flexibacter*, using a dye binding method (Kean 1968). To remove interfering phospholipids and lipids containing ester sulfate, the lipids must be acid hydrolyzed. Unfortunately, the dye failed to react with capnine, the form of sulfonolipid in the lipid extract after hydrolysis. Thus, it is not known what proportion of the total biomass was composed of cells belonging to the genera *Cytophaga* and *Flexibacter*, only that the population incorporating ^{35}S -sulfate into sulfonolipids was approximately 1 % of the total biomass, as estimated by phospholipid-P.

Although the addition of earthworms to soil had little measurable effect on total biomass as indicated by phospholipid-P and analysis of PL-FAMES, the dynamics of ^{35}S -sulfonolipids was altered by their presence. The content of ^{35}S -labelled sulfonolipids in soil reflects the balance between synthesis and degradation. An initial decrease in ^{35}S -sulfonolipids suggested decomposition of ^{35}S -labelled cells of *Cytophaga* and *Flexibacter* during passage of the soil through the earthworm's gut. This was followed by a stimulation in cell growth, either in the casts or in the soil disturbed by the earthworms, which may account for the observed increases in plateable microorganisms in earthworm casts (Parle 1963; Shaw and Pawluk 1986). The most striking difference in ^{35}S -sulfonolipid dynamics between the check and worm-worked soils was the apparent

stabilization of the active population in the worm-worked soil at a level 1/3 greater than the check soil between 40 and 50 days. Thus, the activities of earthworms may not increase the total microbial biomass but may stimulate subsets of the biomass within the soil through modification of the microbial habitat.

Conclusions

In summary, sulfonolipids were completely recovered from cells added to soil, and sulfonolipids did not persist in the soil in an extractable form upon cell death. Application of the technique to soil influenced by the activities of the endogeic earthworm *Aporrectodea turgida* demonstrated three phases of ^{35}S -sulfonolipid dynamics: accumulation, steady state and reduction, and is consistent with the expected pattern for a biomarker sensitive to changes in the biomass of *Cytophaga* and *Flexibacter* spp. in soil. The physical disruption of the soil by the activities of earthworms did not appear to alter either the size or gross composition of the microbial biomass based on phospholipid-P and analysis of PL-FAME profiles. Instead, there appeared to be a stimulation of subsets of the biomass as illustrated by the altered dynamics of ^{35}S -sulfonolipids. It would be instructive to know which other genera were stimulated or disadvantaged by the activities of earthworms and the physiological potential of these genera.

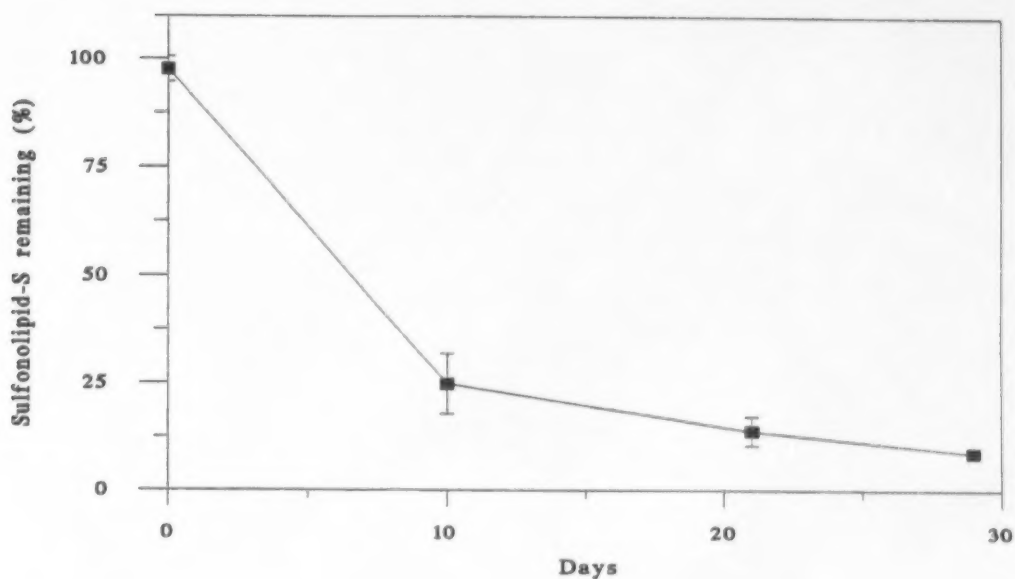


Figure 5.1. Recovery and persistence of ^{35}S -labelled sulfonolipids added within whole cells (late log phase) of *Cytophaga hutchinsonii* to soil. Bars are sample standard deviations of the mean, $n = 3$ at 0 days; $n = 4$ at 10, 21 and 29 days.

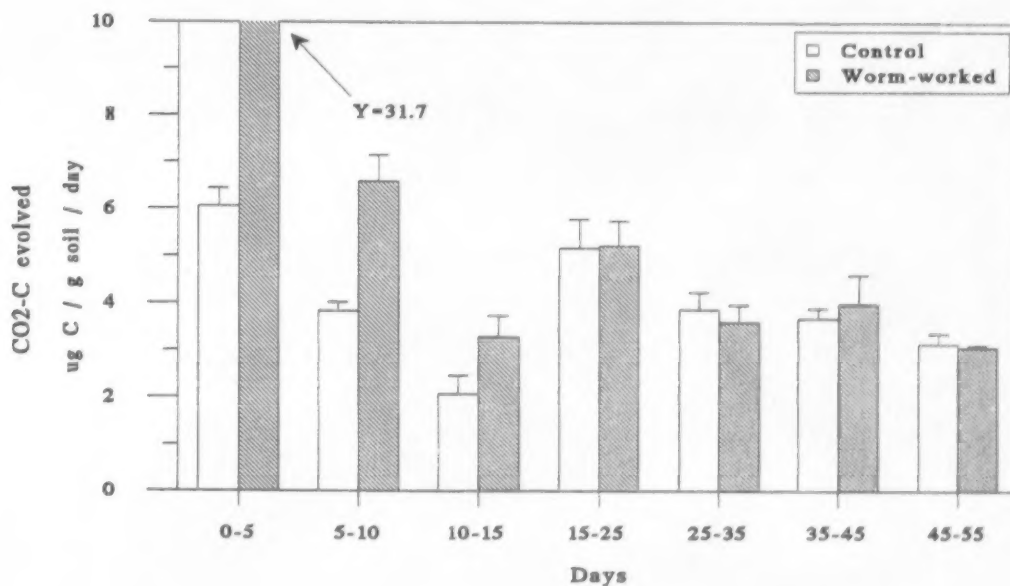


Figure 5.2. Rate of CO_2 evolution from soil microcosms with and without the earthworm *Aporrectodea turgida*. Worms removed on day 5. Bars are sample standard deviations of the mean, $n = 4$.

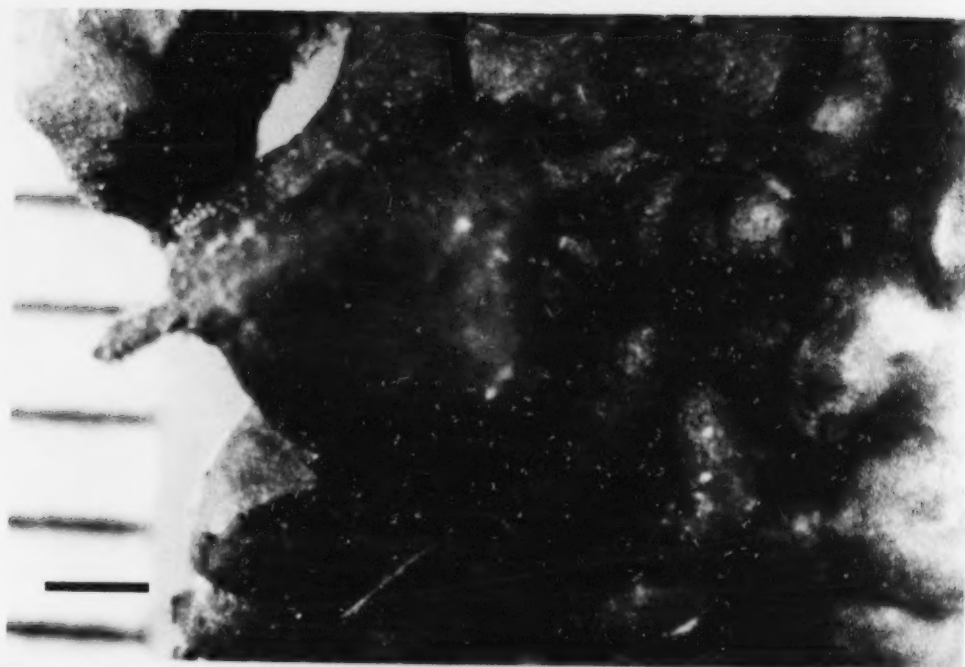


Figure 5.3. Modexi from *Aporrectodea turgida*, age = 50 days. Bar = 1 mm

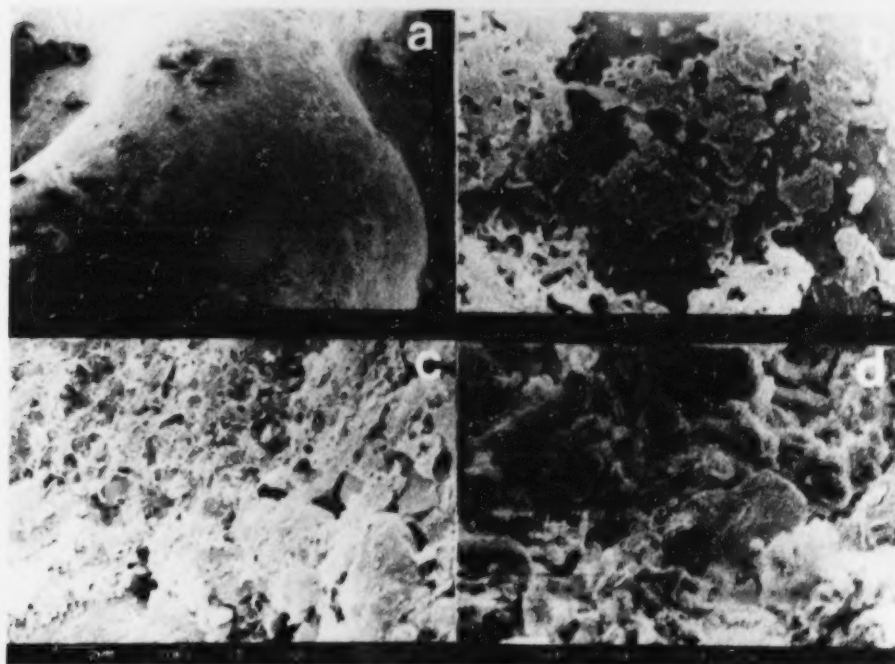


Figure 5.4. Scanning electron micrograph of modexi from *Aporrectodea turgida*. (a) cast surface, age = 15 days, bar = 200 μ m; (b) & (c) close up of (a), bar = 2 μ m. (d) interior of cast (fracture surface), age = 1 day, bar = 20 μ m.

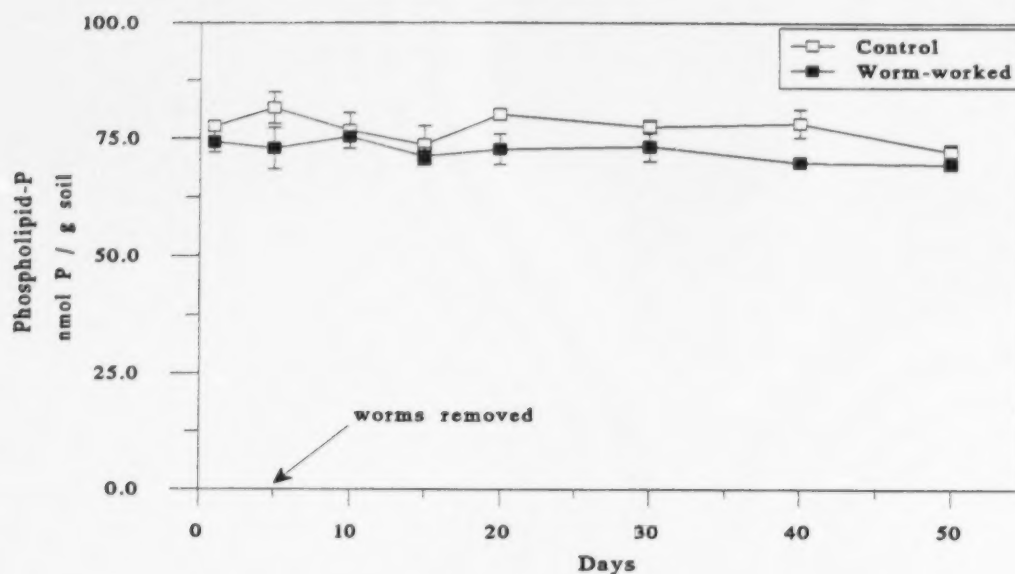


Figure 5.5. Phospholipid-P content of soil worked by the earthworm *Aporrectodea turgida*. Bars are sample standard deviations of the mean, $n = 2$ for check soil; $n = 3$ for worm-worked soil.

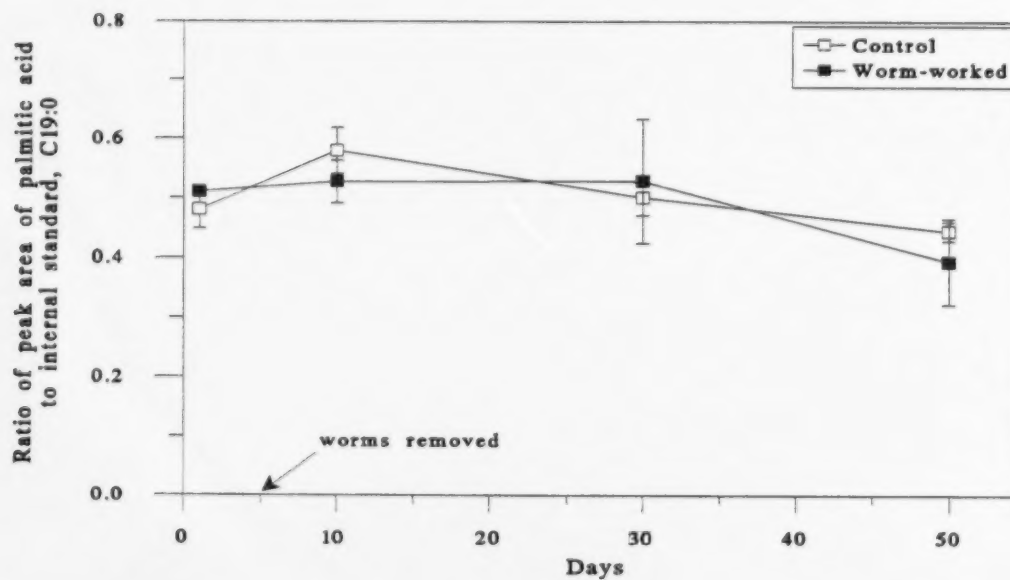


Figure 5.6. Ratio of peak areas of palmitic acid (C16:0) to the internal standard methyl-nonadecanoate (C19:0). Bars are sample standard deviations of the mean, $n = 2$.

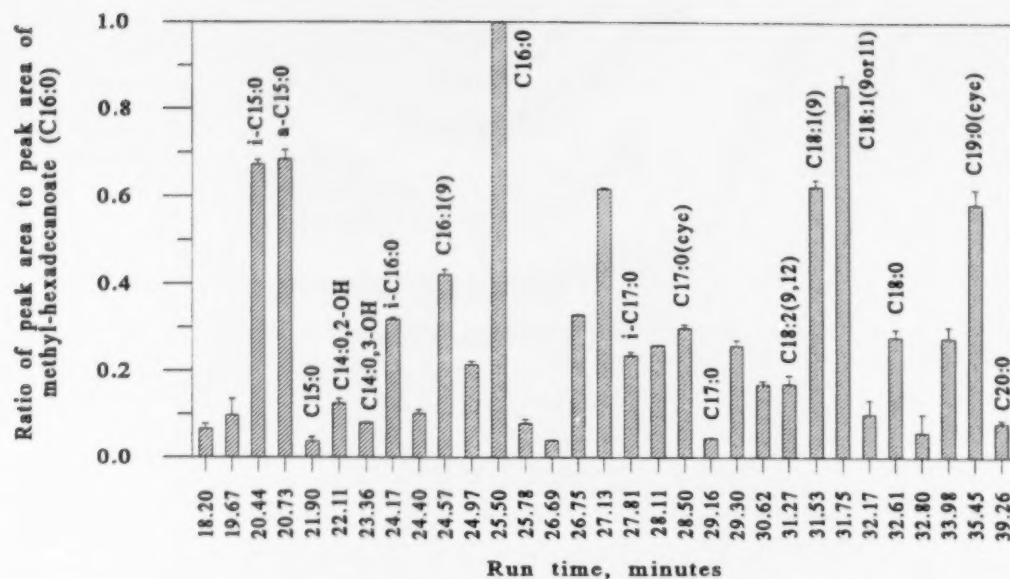


Figure 5.7. Distribution of PL-FAMES in soil without earthworms on day 1. Values are ratios of chromatogram peak areas normalized to the most abundant FAME in the sample, methyl-hexadecanoate (palmitic acid). Identified FAMES confirmed by GC-MS. Error bars are sample standard deviations of the mean, $n = 2$.

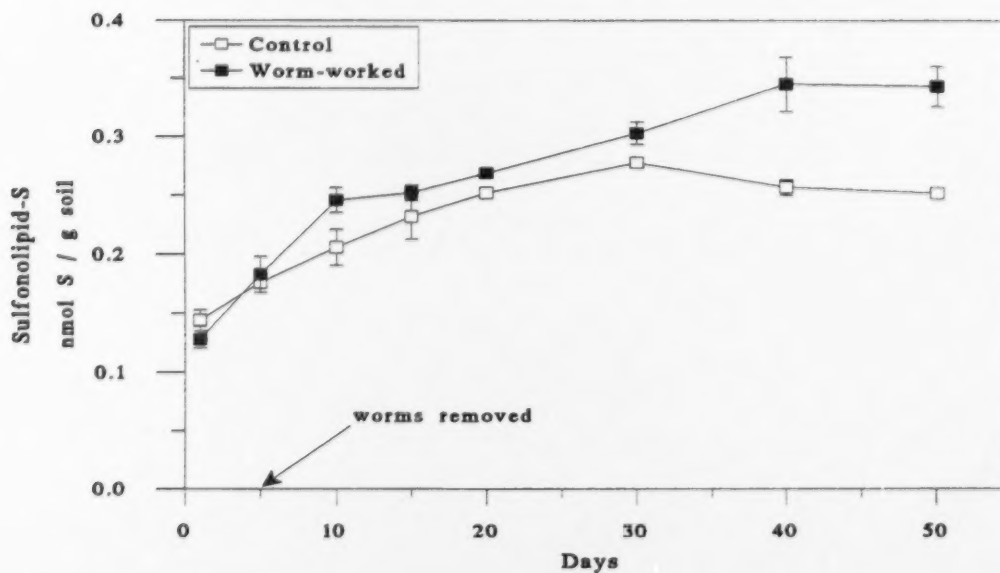


Figure 5.8. ^{35}S -labelled sulfonolipid content of soil worked by the earthworm *Aporrectodea turgida*. Bars are sample standard deviations of the mean, $n = 2$ for check soil; $n = 3$ for worm-worked soil.

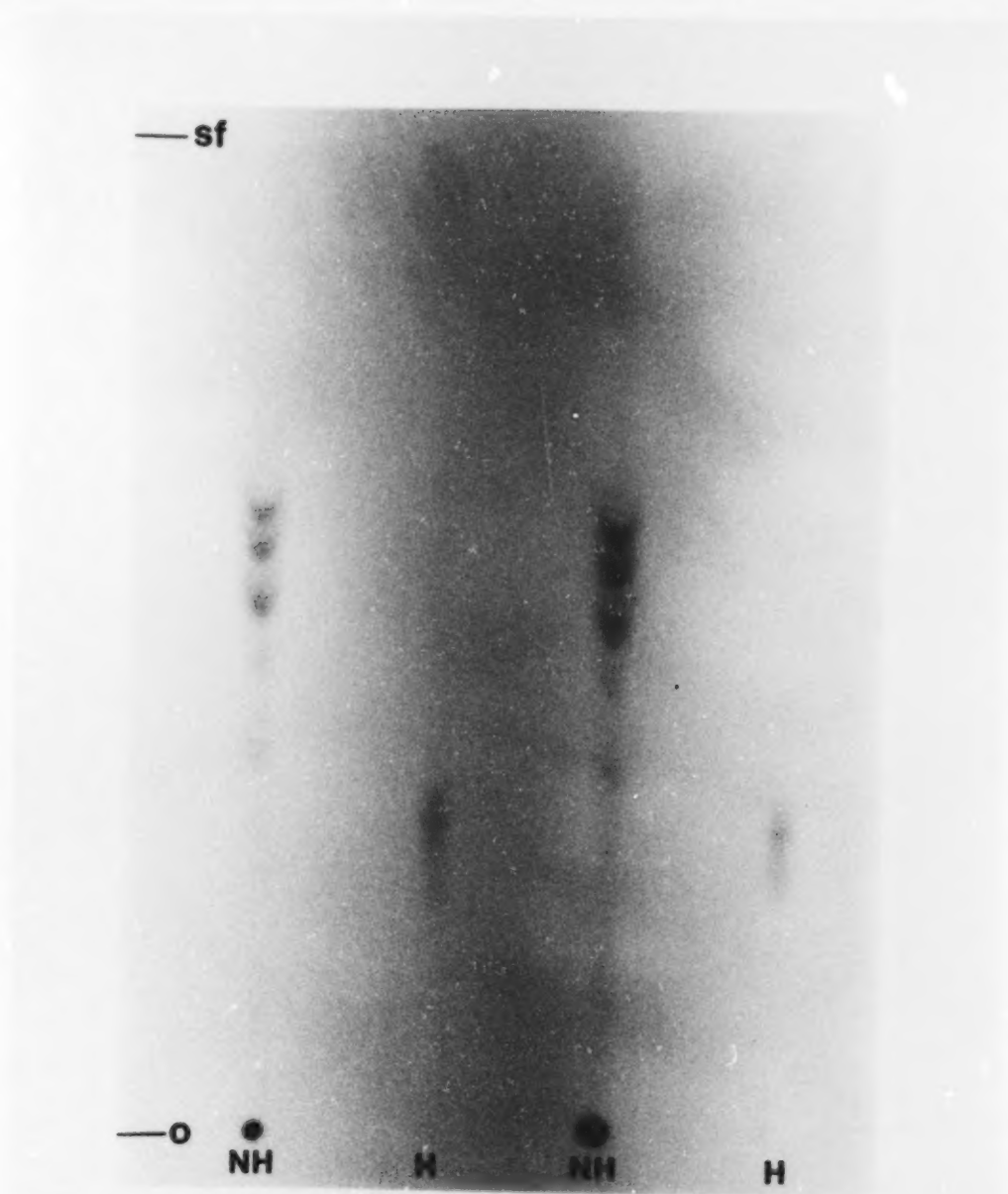


Figure 5.9. Autoradiogram of nonhydrolyzed (NH) and acid/base hydrolyzed (H) ^{35}S -labelled lipids extracted from soil worked by the earthworm *Aporrectodea turgida* and separated by thin-layer chromatography. O = origin; Sf = solvent front.

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CHAPTER 6

SYNTHESIS

Dissertation research should reflect work that is "original, substantial, significant and independently carried out" (Council of Graduate Schools 1991). The seed for my dissertation began with the notion of faunal excrement, particularly earthworm fecal pellets, providing a unique microbial habitat in soil. Reports of enriched numbers of platable *Cytophaga* spp. in earthworm fecal pellets (Shaw and Pawluk 1986) coupled with a phrase in a review article linking unusual sulfonolipids to the genus *Cytophaga* (White 1986) led to an investigation of sulfonolipids as a potential biomarker for the gliding bacteria belonging to the genera *Cytophaga* and *Flexibacter* in soils. Progress towards this goal began with the isolation from soil of several members of the genera *Cytophaga* and *Flexibacter* from which the suitability of sulfonolipids as a biomarker was established. Sulfonolipids were then used to study the population dynamics of *Cytophaga* and *Flexibacter* spp. in soil *in situ* as influenced by the activities of the geophagic earthworm, *Aporrectodea turgida*. Thus, I propose to show that my dissertation thesis constitutes an original contribution to knowledge and is both substantial and significant in terms of our understanding of microbial ecology in soils *in situ*.

Difficulties encountered while trying to isolate members of *Cytophaga* and *Flexibacter* from soil highlighted both the physiological and morphological diversity within these two genera and the unsuitability of cultural and microscopic methods for their enumeration in soils. First, the inability to purify cellulolytic cytophagas from enrichment cultures by streaking onto glucose agar suggested that soil dwelling isolates may be unable to use simple sugars upon initial isolation. This led to the development of the "back-door" method which permitted isolation and purification of cellulolytic cytophagas from soils using a cellulose-based medium as the only source of carbon (Chapter 2). Second, the diagnostic gliding motility of these bacteria, as illustrated by characteristic swarming on agar media, was very dependent on nutrient concentration. At too high or too low nutrient concentrations swarming was not observed on agar media, and compact colonies typical of most eubacteria were formed. Furthermore, gliding was not observed for two isolates, CyG3, a *Cytophaga* sp. and G1 (uncertain taxonomic affiliation), under any of the conditions tested. Third, the cyclic change in cell shape typical of *Flexibacter* spp. was highly dependent on media composition and nutrient

concentration. Thus, by substituting media conducive to the expression of these properties for standard nutrient media, the isolation and tentative identification of *Cytophaga* and *Flexibacter* spp. can be greatly simplified.

Sulfonolipids as a biomarker : merits and limitations

The evaluation of sulfonolipids as a biomarker for *Cytophaga* and *Flexibacter* spp. in soil against criteria set forth by Jenkinson and Ladd (1981) has been successfully completed by establishing that (1) from the literature, sulfonolipids, specifically capnoids, are unique to organisms of the genera *Cytophaga* and *Flexibacter*; (2) sulfonolipids could be completely recovered from cells and accurately measured using radiolabelled ^{35}S -sulfate and isotope dilution techniques; (3) sulfonolipid concentration among the noncellulolytic isolates, with the exception of one isolate, spanned a narrow range (40-60 $\mu\text{mol-S/g}$ cell protein); and, (4) this range in sulfonolipid concentration was maintained under differing growth conditions. Furthermore, sulfonolipids were completely recovered from cells of *Cytophaga hutchinsonii* added to soil and did not persist in the soil in an extractable form upon decomposition of the cells. Although the evaluation of sulfonolipids as a biomarker for *Cytophaga* and *Flexibacter* spp. in soils was not exhaustive, the extent of evaluation reported herein extends that in the literature. Such a comprehensive evaluation was possible only because of the specificity of sulfonolipids to the genera *Cytophaga* and *Flexibacter*, in contrast to other lipid biomarkers such as phospholipid-P and ergosterol which encompass broad taxonomic groups. Signature fatty acids may have a similar specificity; however, the same rigor has not been applied to studies evaluating their potential as quantitative biomarkers. Nevertheless, this does not negate the use of signature fatty acids to indicate qualitative changes in microbial communities in environmental samples.

A potential limitation to the use of sulfonolipids as a biomarker for *Cytophaga* and *Flexibacter* spp. in natural environments stems from the necessity of labelling the sulfonolipid pool with radioactive sulfur. Because chemical standards for sulfonolipids are not commercially available, the most reliable method to determine their presence in lipid extracts was through labelling them with radioactive sulfur. This, in conjunction with autoradiography to ascertain their chromatographic mobility (R_f) on thin-layer plates, allowed unequivocal identification of the sulfonolipids, N-acylcapsine and capnine.

Accurate assessment of sulfonolipid content, however, requires confidence in the specific activity of ^{35}S -sulfate accessible to the growing cells. This is not a concern for cells grown in a homogeneous and defined liquid medium where all sources of sulfur are known, but becomes important for growth of cells in soil. First, possible changes in the amount of sulfate through mineralization and immobilization of sulfate required the ^{35}S -sulfate to be added to soil in a carrier of nonradiolabelled sulfate. Careful consideration was given to balancing the amount of carrier sulfate required to offset changes in the sulfate pool without resorting to excessive amounts of added radioactivity while avoiding losses in analytical sensitivity due to lowered specific activities. Second, the potential for unequal distribution of the added sulfate label within the soil can be balanced by sampling an adequate soil volume. A third concern, the ability of some isolates to incorporate sulfur from protein into sulfonolipids while confronted with excess inorganic sulfate, could result in sulfonolipid contents based on the specific activity of sulfate in soil to be underestimated. In light of the degradative abilities of these organisms, such a situation is highly probable, but as of yet unconfirmed in soil.

The advantages gained by using radiolabelled sulfur for developing sulfonolipids as a biomarker for *Cytophaga* and *Flexibacter* spp. in soil outweighed any possible disadvantages inherent in isotope dilution techniques. As sulfonolipids were the only sulfur-containing lipids produced by the *Cytophaga* and *Flexibacter* isolates studied herein, methods were readily developed. Confirmation of the presence of capnoids in the lipids extracted from soil was made possible through autoradiographic visualization of ^{35}S -sulfonolipids on thin-layer plates. By optimizing the amount of radioactivity required with the amount of carrier sulfate, and taking into account the length of the experiment and the half-life of ^{35}S , reliable measures of ^{35}S -sulfonolipids could be obtained from 2 g of soil containing a minimum of 0.1 nmol sulfonolipids/g soil. This equates to approximately 2.6×10^7 cells the size of *C. hutchinsonii* /g soil based on factors of 52.6 $\mu\text{mol-S/g}$ cell protein (Chapter 3) and 7.4×10^{-14} g protein/cell of *C. hutchinsonii* (data not shown). This sensitivity is comparable to other biochemical markers assayed by gas chromatography or high performance liquid chromatography (White 1983).

Because of the dichotomy in sulfonolipid content between noncellulolytic and cellulolytic cytophagas (Chapter 3) there is a degree of uncertainty in assigning a conversion factor to equate sulfonolipid content to biomass of *Cytophaga* and *Flexibacter* cells. The difficulties associated with isolation of cellulolytic cytophagas from soil makes it unlikely that the proportion of biomass attributable to cellulolytic cytophagas can be determined culturally. Nevertheless, potential exists for ascertaining

the proportion of cellulolytic cytophagas in soil through examination of the N-acyl fatty acid component of sulfonolipids. To date, only four isolates have been examined for their N-acyl residues, and none of these was a cellulolytic cytophaga (Godchaux and Leadbetter 1983). The presence of a 2-hydroxy fatty acid group in the sulfonolipids of *C. hutchinsonii* instead of the more common 3-hydroxy fatty acid group is promising in this regard. A second possibility rests upon whether the structural variations in the capnine core of *C. hutchinsonii* are characteristic of all cellulolytic cytophagas. Thus, further structural studies on the capnine core and attached N-acyl residues are warranted and may provide some taxonomic specificity. An alternative approach would be to seek a genetic probe specific to cellulolytic cytophagas, and enumerate their biomass in soil through the polymerase chain reaction.

The efficiency of the chloroform-methanol solvent in extracting lipids from the cells was evaluated by adding ^{35}S -labelled cells to soil then measuring the recovery of ^{35}S -labelled sulfonolipids from those cells over time. Implicit in this approach is the assumption that the added cells end up in microsites identical to cells grown in soil *in situ*. From studies of plasmid transfer between donor and recipient populations of pseudomonads, this assumption may not be valid as the frequency of transfer was highly dependent on the method of introduction of donor and recipient cells. Plasmid transfer did not occur when donor and recipient cells were mixed separately into soil which was then combined and further mixed suggesting the bacterial strains inhabited spatially separated niches with little movement of bacteria under the constant environmental conditions (Kuikman et al. 1990; van Elsas et al. 1990). Furthermore, added cells may accumulate on the surfaces of aggregates or within easily accessible pores. Despite these limitations, the extracting solvent appears to either (1) completely recover lipids from cells within the soil from biomass estimates based on phospholipid-P (Chapter 5), or (2) is reproducible in its recovery of lipids based on standard deviations of extractable ^{35}S -sulfonolipids (Chapter 5).

Implicit in the use of radioisotopes is the incorporation of the radioactive label only into cells which are actively growing. Analysis of sulfonolipids by high performance liquid chromatography would allow the total population of *Cytophaga* and *Flexibacter* spp. to be measured in environments outside the laboratory; however, differentiation between active and resting populations is not possible without the use of radioisotopes. Thus, the increase and decrease in net ^{35}S -sulfonolipids observed in the check soil (Chapter 5), which was taken as confirmation of the sensitivity of the biomarker to changes in the active biomass of *Cytophaga* and *Flexibacter* spp., may have been masked by a much larger background of sulfonolipids in the nongrowing population.

Microbial dynamics in soil worked by the earthworm *Aporrectodea turgida*

The application of lipid biomarker techniques to the study of microbial communities in soil worked by the earthworm *Aporrectodea turgida* presented a picture conceptually different from past studies that were based on platable organisms (Chapter 5). First, passage of the soil through the earthworm did not result in increased biomass in soil containing freshly deposited casts. In fact, as this and recent other studies indicate (Scheu 1987; Daniel and Anderson 1992), biomass may actually decrease in the casts due to consumption of the microbiota by the earthworm. Second, analysis of phospholipid-linked fatty acid methyl esters detected no significant alterations in community structure within the soil worked by the earthworm; although, future analysis of isolated cast material may uncover some changes masked by the soil not passed through the earthworm's gut. This concept is truly intriguing as it suggests that most of the soil biota passes through the earthworm gut unchanged. Given the physical disruption of the soil during passage through the earthworm gut to emerge as a soil paste, where does this protection of cells arise? The ultrastructure of earthworm casts from mineral soil horizons suggests living microorganisms exist in "protected sites, either with plant debris, within soil microaggregates, or as clay-coated microcolonies Ingested microaggregates often appear to be intact and hardly modified by passage through the gut" (Lee and Foster 1991). Fluorescent staining of ultrathin sections made from earthworm casts supports passage of intact microaggregates up to 200 μm in diameter, with larger cells, presumably recently active, being concentrated in streaks between soil material and in voids (Altemüller and Joschko 1992).

Passage of soil through earthworms often involves comminution of plant materials and increased mineralization of nutrients. If we consider earthworms as reorganizers of soil material, earthworms may function to alter the distribution of substrates and microorganisms in both time and space. Hence, upon gut passage some communities will find themselves sequestered within microsites devoid of substrates or conversely, surrounded by a feast, while most microorganisms, particularly those residing within microaggregates, find little alteration in their surroundings, and exist as before relying on substrates diffusing to their microsites. Evidence to support the changing status of microsites upon passage of soil through earthworms comes from the altered population dynamics of *Cytophaga* and *Flexibacter* spp. in soil worked by the earthworm *Aporrectodea turgida* (Chapter 5).

Sulfonolipids: future applications

To further our understanding of the ecology of *Cytophaga* and *Flexibacter* spp. in soil future endeavors should include:

(1) the development of a method using high performance liquid chromatography, or alternatively, employment of a gas chromatograph equipped with a sulfur detector, to measure total sulfonolipids, which in conjunction with radioisotopes, would allow the turnover of naturally occurring *Cytophaga* and *Flexibacter* spp. to be measured in soil. An experiment is being planned which would allow the actual growth and death rates of the population to be measured in soil instead of the net growth measurements reported in Chapter 5.

(2) attempts to discriminate between functionally different species of *Cytophaga* and *Flexibacter*. In particular, could the N-acyl component of sulfonolipids or the capnine core be used to separate cellulolytic from noncellulolytic cytophagas, as discussed previously?

(3) a further investigation of the aberrant cells of *C. hutchinsonii*, both morphologically and physiologically, and to determine the universality of these features among other *Cytophaga* and *Flexibacter* spp.

(4) the application of sulfonolipids to other environments, particularly the rhizosphere, where the gliding motility and degradative capabilities of these bacteria would suit them well.

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Appendix A: Conversion factor for protein assays

Protein was determined by the method of Lowry et al. (1951) except for cultures containing cellulose powder where the dye-binding method of Bradford (1976) was used. Glucose, released from the cellulose powder during heating with NaOH, increases color development in the Lowry method (Bradford 1976; personal observation). To compare protein contents of cell cultures, cultures grown in basal medium plus glucose were assayed by both methods using bovine albumin as the standard. The molar absorbance of bovine albumin was not consistent between the two assays, therefore, protein contents of the cellulolytic isolates grown on cellulose powder and assayed by the method of Bradford needed to be converted to Lowry protein equivalents using the factor Lowry/Bradford = 1.55 determined for *Cytophaga hutchinsonii*.

Isolate	Protein, mg/mL		Lowry/Bradford
	Lowry	Bradford	
CyS1-R1	141.81	98.78	1.44
CyS1-R2	152.19	100.85	1.51
CyS1-R3	149.09	95.24	1.57
CyS1-R4	149.09	94.99	1.57
			X = 1.52
			Sx = 0.06
CH-R1	213.38	132.28	1.61
CH-R2	180.42	120.01	1.50
CH-R3	215.25	140.34	1.53
CH-R4	218.21	133.98	1.63
			X = 1.55
			Sx = 0.06
FxG4-R1	123.97	95.2	1.30
FxG4-R2	133.72	107.11	1.25
FxG4-R3	137.86	107.10	1.29
FxG4-R4	130.06	106.08	1.22
			X = 1.27
			Sx = 0.04

Cy = *Cytophaga*; Fx = *Flexibacter*; CH = *C. hutchinsonii*; R = rep. #

Appendix B: Calibration of azure-A dye method

For isolates grown in the presence of tryptone and inorganic sulfate (TGY medium), the total cellular content of sulfonolipids was determined by reaction with azure-A dye (Kates 1986). Bovine sulfatide was used as the standard to calculate sulfonolipid-S. Because the molar absorbance of bovine sulfatide and sulfonolipids may differ, the azure-A method was calibrated using sulfonolipids extracted from isolates grown in the presence of inorganic sulfate only. Addition of ^{35}S -sulfate to the growth medium allowed the actual sulfonolipid content of the isolates to be determined by isotope dilution.

Isolate	Sulfonolipid-S, μmol		Isotope/Azure A
	Isotope dilution	Azure A	
CyS1-R1	1.47	1.28	0.64
CyS1-R2	1.36	1.67	0.81
FxG4-R1	0.99	1.41	0.70
FxG4-R2	1.05	1.38	0.76
			X = 0.73
			Sx = 0.07

Cy = *Cytophaga*; Fx = *Flexibacter*; R = rep. #

Appendix C: Structure of N-acylcapnine isolated from *C. hutchinsonii*

A. Mass spectrum obtained by negative fast atom bombardment of N-acylcapnine and capnine released by acid methanolysis.

The mass spectrum of N-acylcapnine obtained by negative fast atom bombardment (-FAB) gave a single molecular ion at $m/z = 642$ (Figure 3.1a), suggesting a single fatty acid species in amide linkage with capnine. This was confirmed by gas chromatography-mass spectrometry (GC-MS) of the fatty acid methyl esters (FAMES) released by acid methanolysis by (Part C). A molecular ion at $m/z = 300$ for the FAME recovered after acid methanolysis equates to a fatty acid with molecular weight 269. Subtracting 269 from 642, the molecular weight of N-acylcapnine, gives 373 as the molecular weight of capnine. In the mass spectrum of N-acylcapnine, the most abundant fragment was $m/z = 374$, implying cleavage at the amide bond. Chemical deacylation of N-acylcapnine to form capnine produced a molecular ion at $m/z = 374$ (Figure 3.1b), confirming the molecular mass of capnine from *C. hutchinsonii* as being 23 mass units higher than the molecular mass of 351 reported by Godchaux and Leadbetter (1980; 1984).

B. Infrared spectrum of N-acylcapnine.

The infrared spectrum of N-acylcapnine was consistent with the spectrum obtained for N-acylcapnines isolated from *Flexibacter* sp. strain FS-1 (Godchaux and Leadbetter 1984) with one exception: a prominent absorption peak at 970 cm^{-1} indicating the presence of *trans*-double bonds (Figure C1). The location of the double bond(s) must lie in the parent molecule, capnine, since the fatty acid released upon acid methanolysis of N-acylcapnine lacked double bonds (Part C). Prominent absorption bands were present in the sulfonate ($1058\text{--}1202\text{ cm}^{-1}$) and amide (1532 and 1631 cm^{-1}) regions. A sharp doublet at 1366 and 1384 cm^{-1} confirmed the presence isopropyl groups, either within the capnine core and/or the N-acyl group.

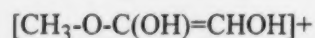
C. Gas chromatography-mass spectrometry of fatty acid methyl esters released by acid methanolysis of N-acylcapnine.

Acid methanolysis of N-acylcapnine followed by gas chromatography of the FAMES released indicated that only one fatty acid was present in N-acylcapnine (Figure C2). The retention time (RT) was 31.51 minutes. This is consistent with the single molecular ion detected by -FAB mass spectrometry of N-acylcapnine. A common practice to ensure all fatty acids released by acid methanolysis are completely methylated is to further methylate them with BCl_3 in methanol (Fautz et al. 1979; Godchaux and

Leadbetter 1984). The appearance of several additional peaks in the gas chromatogram coupled with tailing of the peak at RT = 31.51 suggested degradation and the production of artifacts during methylation with BCl₃ (Figure C3). This leads one to suspect the validity of FAME analysis where this procedure has been used (Fautz et al. 1979; Godchaux and Leadbetter 1984).

The fatty acid released from N-acylcapsine by acid methanolysis was identified by GC-MS as containing 17 carbons with a hydroxyl group on carbon two and terminal methyl branching [*i*C17:0 (2-OH) (Figure C4) based on the following:

1. A molecular ion present at $m/z = 300$ corresponding to the molecular formula C₁₈H₃₆O₃ (M-methanol (31) = 269). The molecular ion is absent from mass spectra of 3-hydroxy FAMES (Odham and Stenhagen 1972; Fautz et al. 1979)).
2. No peak at $m/z = 91$; thus, no unsaturation (i.e. double bonds) (Odham and Stenhagen 1972).
3. Characteristic peak at $m/z = 90$ (Odham and Stenhagen 1972), corresponding to the rearranged ion:



4. Very small peak at M-50 (molecular ion minus methanol and water), which is prominent in all hydroxylated FAMES except 2-hydroxy FAMES (Ryhage and Stenhagen 1960).

The fatty acyl group *i*C17:0 (2-OH) was not identified in the acid methanolysates of N-acylcapsines isolated from *Capnocytophaga* sp. strain P7, *Cytophaga johnsonae* ATCC 17061, a marine *Cytophaga* sp. strain 7B and *Flexibacter* sp. stain FS-1 (Godchaux and Leadbetter 1984). Neither was this species of fatty acid present in whole-cell methanolysates of *Cytophaga johnsonae* Cyj1 and *Cytophaga* sp. strain samoa, while trace amounts were detected in *Flexibacter elegans* Fx e1 (Fautz et al. 1979). Approximately 10% of the fatty acids in whole-cell methanolysates of three species of myxobacter, *Myxococcus fulvus* Mx f2, *Cystobacter velatus* P1 v9 and *Stigmatella aurantiaca* Sg a1; however, were identified as *i*C17:O (2-OH) (Fautz et al. 1979, 1981). These reports, however, may be suspect since the FAMES were further methylated with BCl₃ (Fautz et al. 1979, 1981; Godchaux and Leadbetter 1984).

There remain three areas of uncertainty in the structure of the capsine core:

1. The origin of the extra mass units.
2. The location and number of double bonds. This will require periodate-permanganate oxidation of capsine and analysis of the resulting FAMES by GC-MS.
3. Confirmation of a hydroxyl group vicinal to the amino group. This will require periodate oxidation of capsine and analysis of the resulting FAMES by GC-MS.

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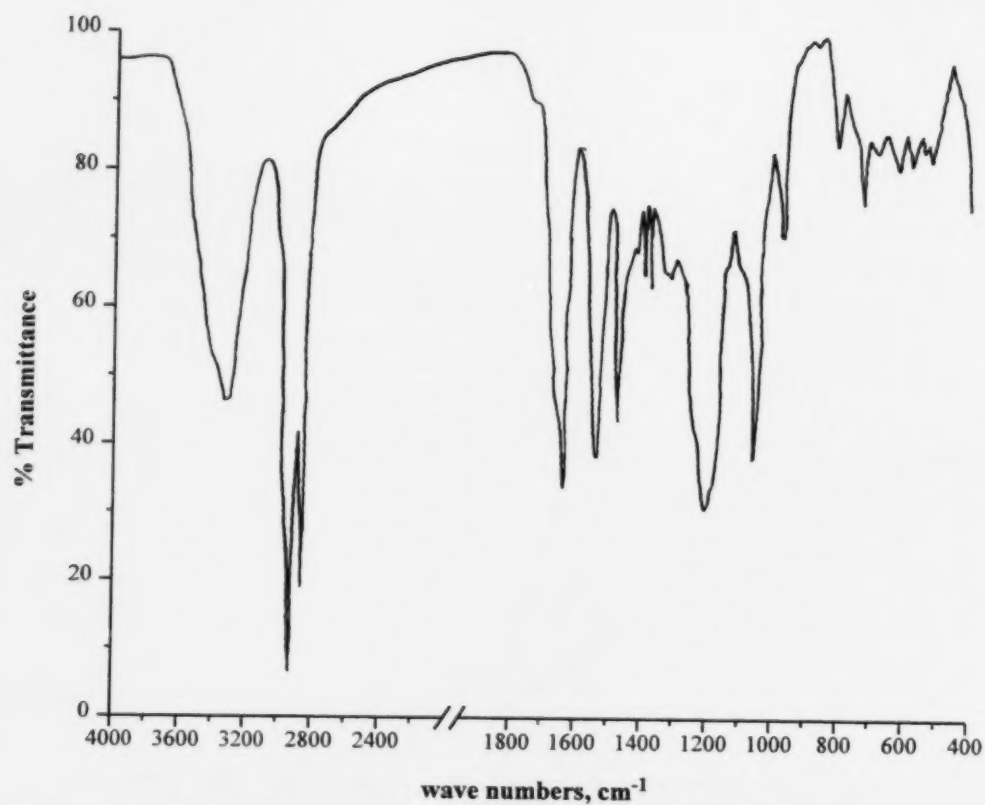


Figure C1. Infrared spectrum of N-acylcapsine isolated from *Cytophaga hutchinsonii*.

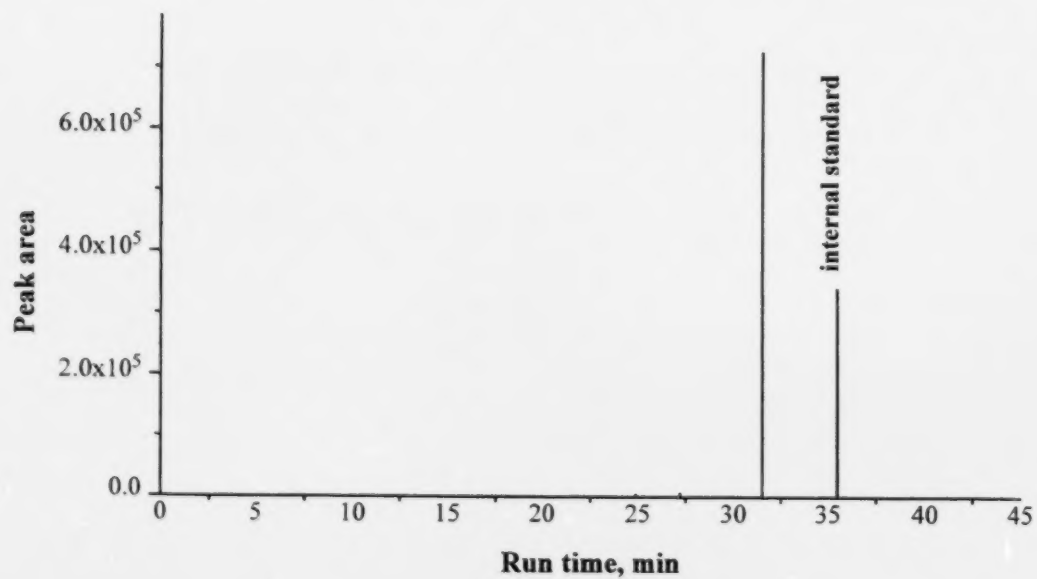


Figure C2. Fatty acid methyl esters released from N-acylcapsine by acid methanolysis and analysed by gas chromatography.

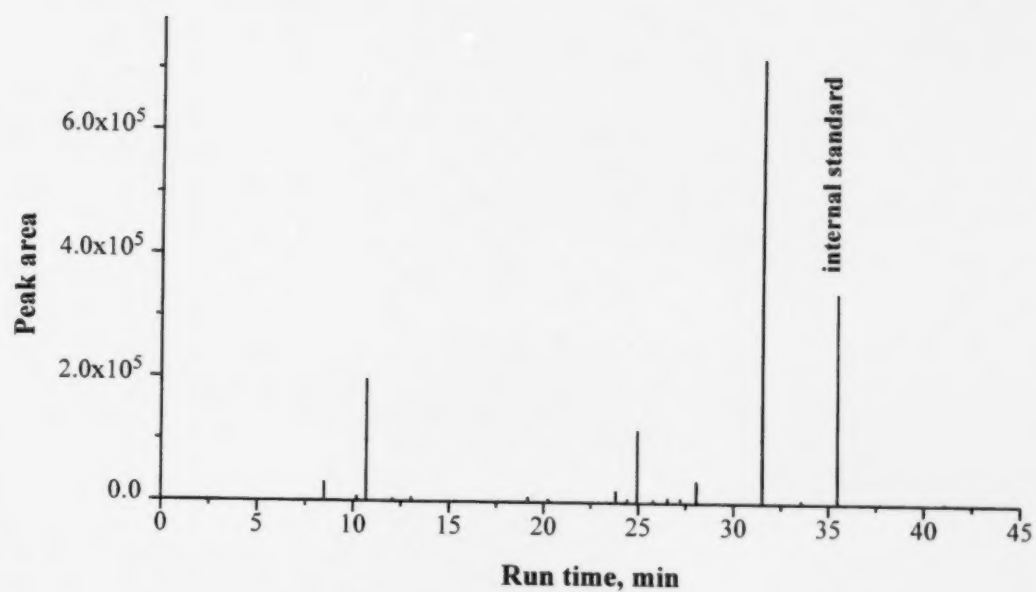


Figure C3. Fatty acid methyl esters released from N-acylcapsine by acid methanolysis, methylated by BCl_3 and analysed by gas chromatography

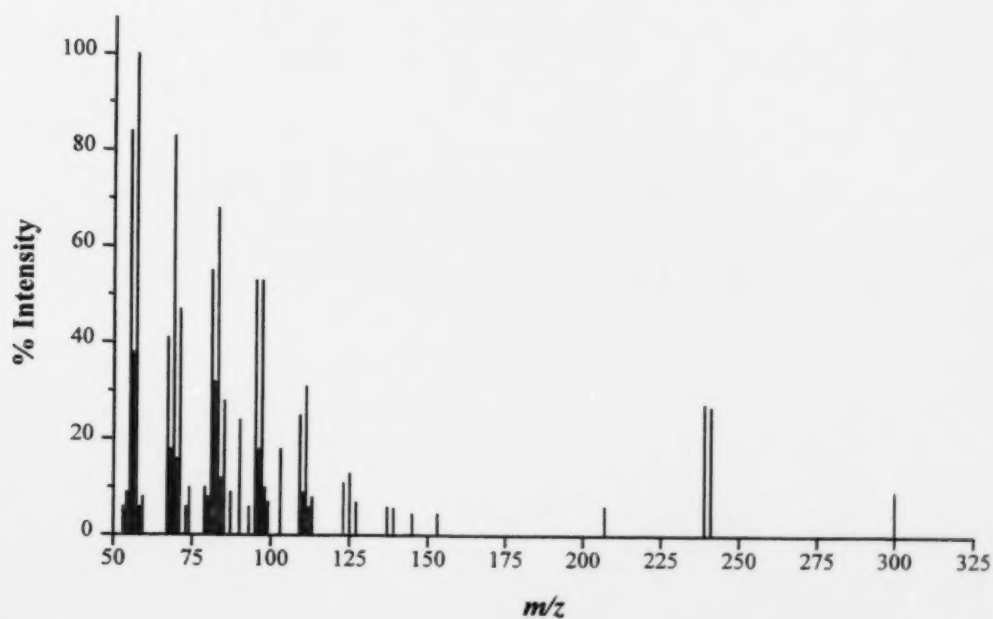
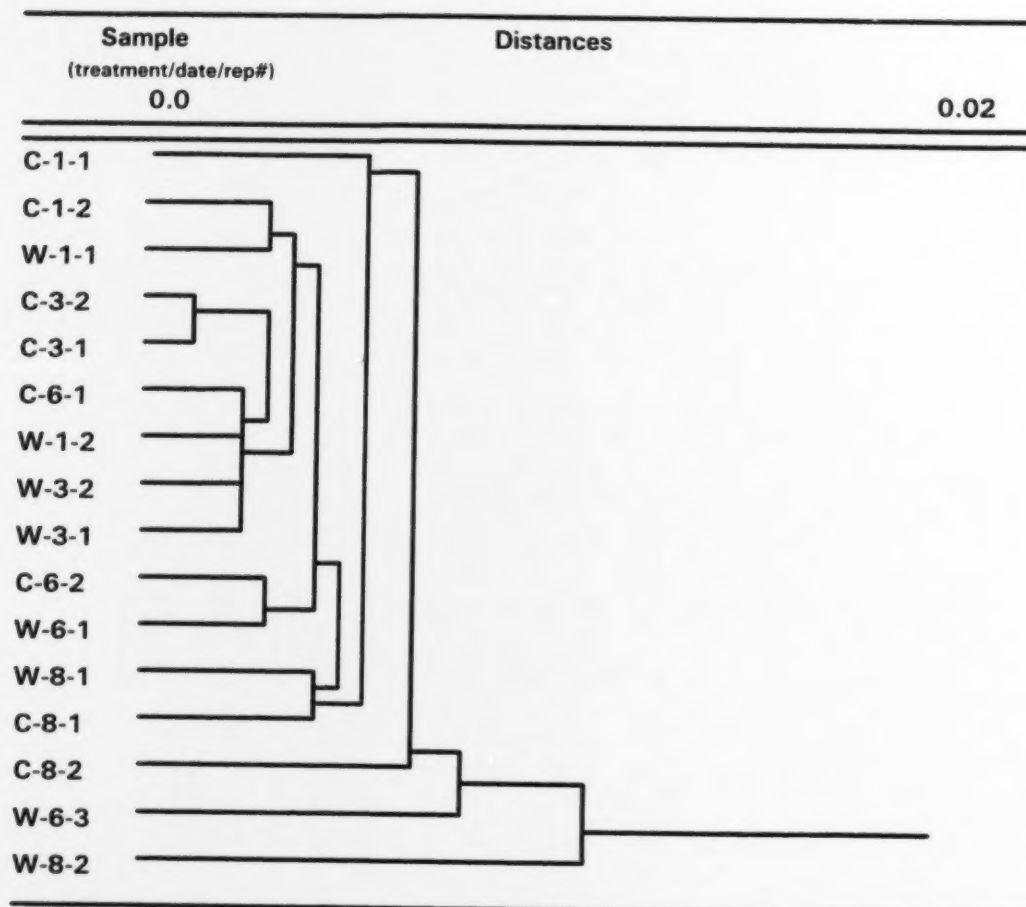


Figure C4. Fatty acid methyl esters released from N-acylcapsine by acid methanolysis and analysed by gas chromatography-mass spectrometry. Mass fragments at < 5 % of maximum intensity not shown.

Appendix D: Cluster analysis of FAMEs

Tree diagram



Treatment: C = check; W = worm-worked.

Date: 1 = 1 day; 3 = 10 days; 6 = 30 days; 8 = 50 days.

Rep #: 2 reps.